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TITLE: Role of Activin A in Immune Response to Breast Cancer

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14. ABSTRACT

In recent years, progress has been made in the development of immune-based therapy for cancer. Conceptually, these treatment strategies have the potential of harnessing the immune system to combat and eliminate cancer cells. One major obstacle to the success of immunotherapy in both human and animal studies is the development of immunologic tolerance in tumor-bearing hosts. Therefore, the immune system fails to recognize cancer cells as dangerous and actively suppresses anti-tumor immune responses. Identification of the underlying mechanisms and the critical players that drive tolerance to the tumor is critical to improve the therapeutic efficacy of immunotherapy. Recent data indicate that activin A, a small protein secreted by some immune cells and by breast cancer cells has immune regulatory functions that may play a key role in promoting escape of tumors from immune control. The proposed studies will test the hypothesis that activin A secreted by breast cancer cells plays a key role in suppressing antitumor immunity. The goals are to demonstrate the role of activin A produced by breast cancer cells in tumor growth and metastasis, and the potential therapeutic benefit of blocking activin A to increase the response to radiotherapy.

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Table of Content

1 Introduction

Owing to its ability to spread systematically, breast cancer remains a life-threatening tumor. Therefore, efforts in developing new treatment strategies are needed in order to eradicate metastatic breast cancer. In this respect, the activation of the immune system to elicit anti-tumor immune responses represents one of the most promising approaches that have recently demonstrated some success in other diseases. However, clinically apparent tumors have already harnessed host mechanisms to prevent immune activation and to induce an immunosuppressive microenvironment hindering immunotherapy-based treatments. As a consequence, the immune system fails to recognize cancer cells as dangerous and actively suppresses anti-tumor immune responses.

Identification of the underlying mechanisms and the critical players that drive tolerance to the tumor is critical to improve the therapeutic efficacy of immunotherapy. Recent data indicate that activin-A, a small protein secreted by some immune cells and by breast cancer cells, has immune regulatory functions that may play a key role in promoting escape of tumors from immune control. The specific hypothesis of this project is that activin-A secreted by breast cancer cells plays a key role in suppressing antitumor immunity. The goals are to demonstrate the role of activin-A produced by breast cancer cells in tumor growth and metastasis, and the potential therapeutic benefit of blocking activin-A to increase the response to radiotherapy (RT).

2 Body

During the first year of this postdoctoral BCRP fellowship, we have shown that tumor-derived activin-A contributes in generating immunosuppression by inducing a tolerogenic phenotype of the dendritic cells as well as enhancing conversion of naïve CD4 T cells into induced regulatory T cells. More importantly, similarly to transforming growth factor-beta (TGFβ), our results suggest that breast cancer cells upregulate activin-A production in response to radiation exposure countering the pro-immunogenic effects of radiotherapy. Since our data support a key role of activin-A in immune tolerance by the tumor in vitro, year 2 and 3 were designed to determine the role of activin-A in vivo.

TASK 2: ***Effect of activin A blockade in vivo on breast cancer immunity***

Task 2.a: Knockdown of activin-A in 4T1

To determine the role of tumor cell-secreted activin-A *in vivo*, derivatives of 4T1 ($4T1^{shInhba}$) cells transduced with a set of plasmid encoding short-hairpin (shRNA) specific for murine *Inhba* have been prepared.

Several approaches to knockdown activin-A were investigated with notably the use a shRNA cloning vector p-GFP-V-RS (OriGene, Figure 1A) and the p-GFP-C-shLenti shRNA (OriGene, Figure 1C) plasmids. Unfortunately, both strategies were unsuccessful because of instability of the construct overtime. Indeed, as shown in Figures 1C and 1D, 4T1 transduced cells gradually lost the GFP signal regardless of the

construct used suggesting that the knockdown of activin-A is not stable.

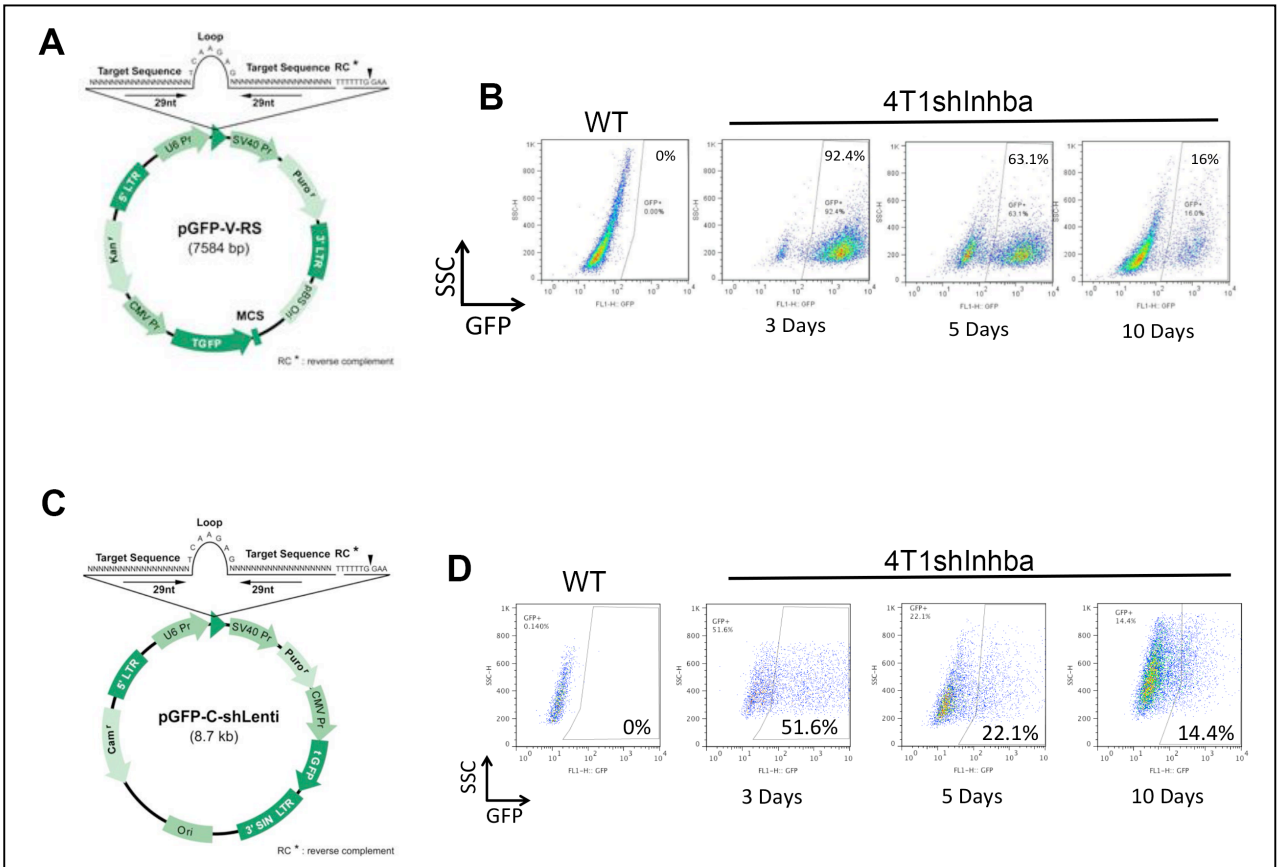

Figure 1: A- Mapping of the shRNA cloning vector pGFP-V-RS. **B**- Representative dot plots of 4T1^{shInhba} GFP+ cells transfected with the pGFP-V-RS vector after 3, 5 and 10 days of culture. **C**- Mapping of the shRNA of the cloning vector pGFP-C-shLenti. D- Representative dot plots of 4T1^{shInhba} GFP+ cells transfected with the pGFP-C-shLenti vector after 3, 5 and 10 days of culture.

To overcome this problem, we decided to modify our strategy and use a pTRIPZ inducible lentiviral shRNA vector (kindly provided by Dr. Robert Schneider from NYU). The pTRIPZ vector is engineered to be Tet-On via the tetracycline response element (TRE) promoter. This equips the pTRIPZ plasmid to provide induced expression of an shRNA in the presence of doxycycline; therefore permitting reversible and inducible gene knockdown. In addition to driving the expression of the shRNA, TRE also drives the expression of a TurboRFP reporter allowing visual tracking of shRNA expression both *in vitro* and *in vivo* (Figure 2).

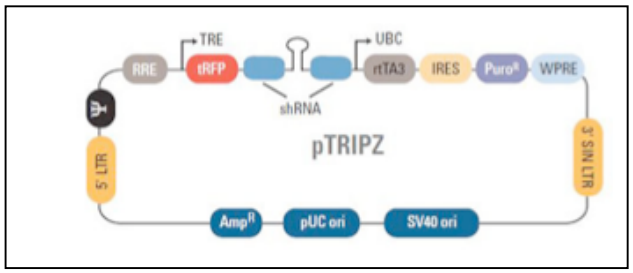

Figure 2: Mapping of the shRNA cloning vector pTRIPZ

First, a set of shRNA contructs targeting different sequences within *Inhba* gene-were inserted in pTRIPZ vector. Because the OriGene sequences were effective at knockdown of *Inhba* gene expression (see previous annual report), we inserted these sequences into the pTRIPZ plasmid (Table 1 – Figure 3).

shRNA ID	Sequence (97-mer)
ShSCR	Scrambled negative control: AATTCTCCGAACGTGTCACGT
ShInhba#1	TGCTGTTGACAGTGAGCGAAACTGTTGCTATCAGAGAAAGTAGTGAAGCCACAGATGTA CTTTCTCTGATAGCAACAGTTCTGCCTACTGCCTCGGA
ShInhba#2	TGCTGTTGACAGTGAGCGCTGGCTGAGAGGATTTCTGTTGTAGTGAAGCCACAGATGTA CAACAGAAATCCTCTCAGCCAATGCCTACTGCCTCGGA
ShInhba#3	TGCTGTTGACAGTGAGCGCCCTTCCACTCAACAGTCATTATAGTGAAGCCACAGATGTA TAATGACTGTTGAGTGGAAGGATGCCTACTGCCTCGGA

Table 1: Sequences of ShInhba gene inserted into pTRIPZ vector.

Figure 3: Alignment results of pTRIPZ sequencing showing that each 97-mer was correctly inserted into pTRIPZ vector.

After obtaining 4T1 cells derivatives containing the 3 different *Inhba* shRNA pTRIPZ, stability of activin-A knockdown *in vitro* was assessed by testing 4T1-derivatives for RFP expression after 15 days of culture. Control cells transduced with scrambled shRNA were similarly tested.
As shown in Figure 4, 4T1 derivatives maintained the RFP signal regardless of the shRNA used suggesting that **the knockdown of activin-A is stable overtime**.

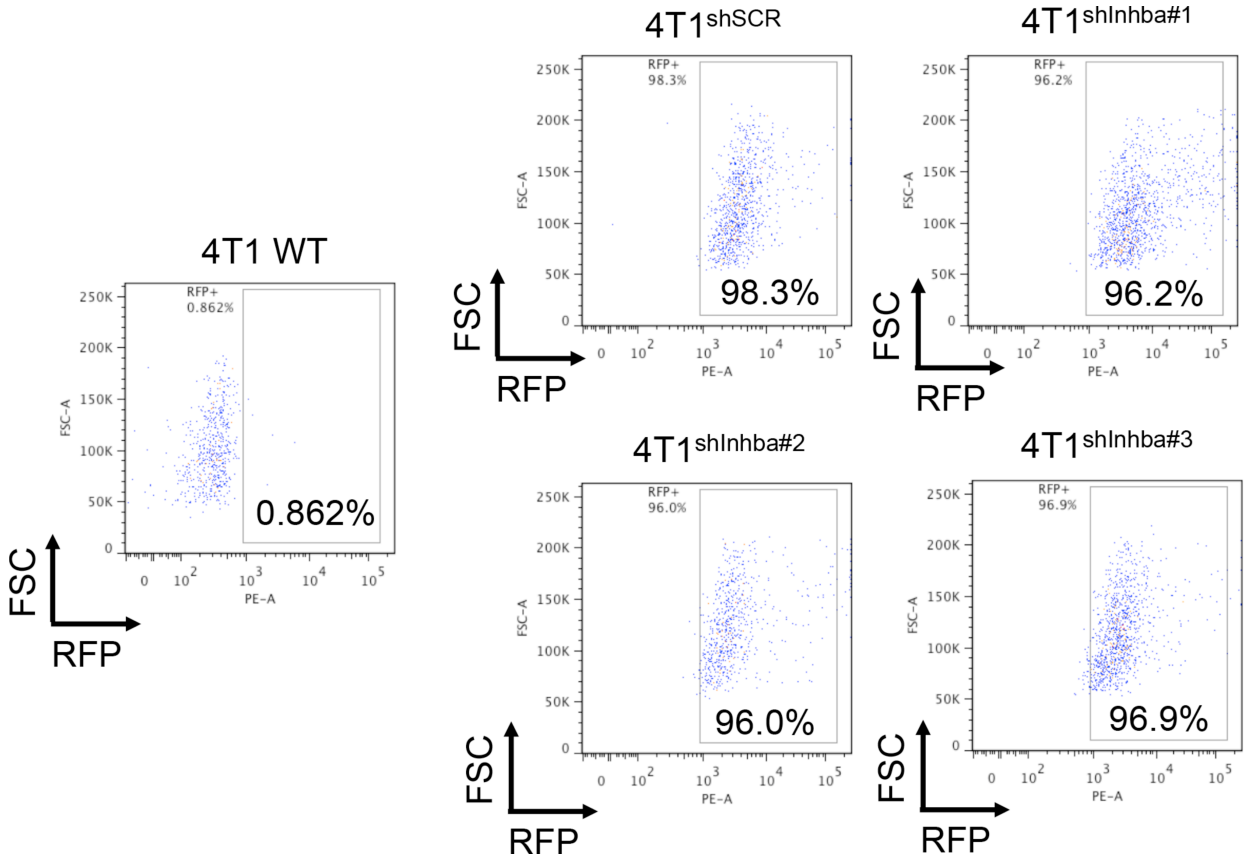

Figure 4: Representative flowcytometry dot plots of 4T1^{shInhba} RFP+ cells after 15 days of culture.

***Task 2.b*: Verification of 4T1 knockdown of activin-A.**

To determine which sequence was most effective at knocking down *Inhba* gene, wild type (WT) 4T1^{WT} or its derivative 4T1^{shSCR}, 4T1$^{shInhba\#1}$, 4T1$^{shInhba\#2}$ and 4T1$^{shInhba\#3}$ were plated and treated with doxycycline for 5 days to induce the expression of the constructs. Quantification of activin-A secreted by tumor cells over 24h was performed by ELISA and RT-qPCR. Results showed that shRNA carrying the sequence #3 was the most effective at inhibiting activin-A secretion by 4T1 cells (Figure 5). Therefore,

4T1$^{shInhba\#3}$ (thereafter 4T1^{shInhba}) were selected for future in vitro and in vivo experiments.

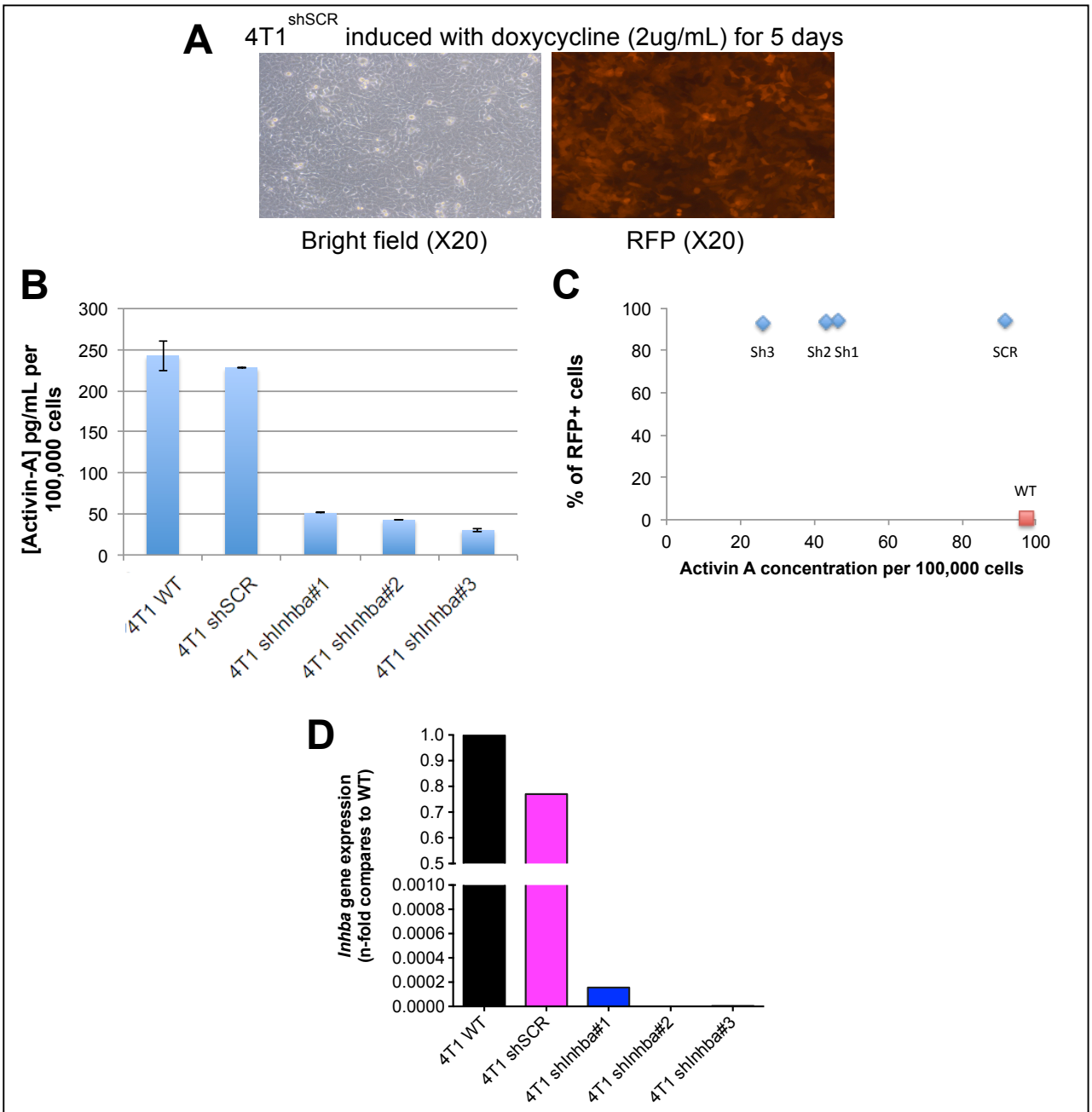

Figure 5: A- Representative pictures of derivatives 4T1 following doxycycline induction. **B-** ELISA quantification of activin-A released by 4T1 and its derivatives. **C-** Activin-A released regarding by the percentage of RFP+ cells. **D-** Gene expression of *Inhba* by RT-qPCR.

In previous in vitro experiments we have shown that activin-A produced by 4T1 cells

was partially responsible for the conversion of naïve CD4 T cells into FoxP3+ induced regulatory (iTreg) T cells. To test the functional consequences of the activin-A knockdown in 4T1 cells we used a transwell system.

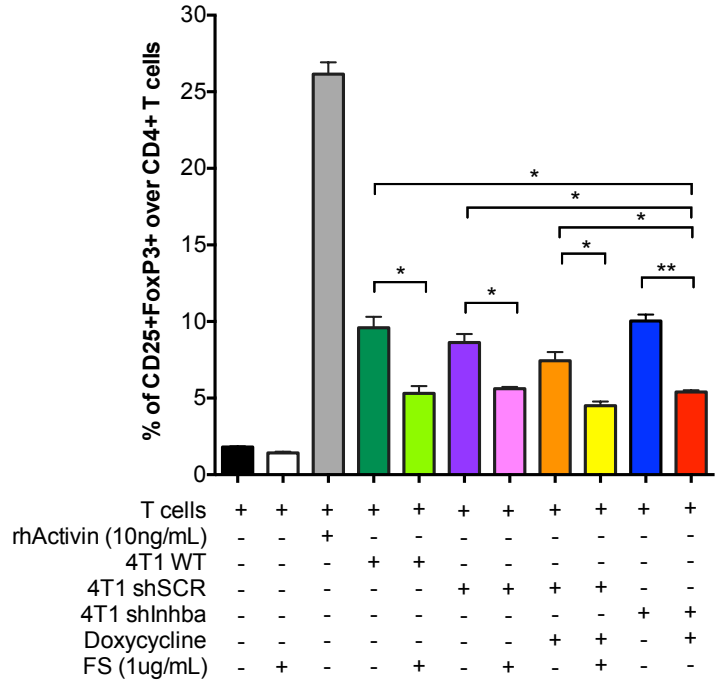

Figure 6: 4T1-tumor derived activin-A convert naïve CD4+ T cells into induced regulatory T cells (iTregs). Naïve T cells were isolated from a Balb/C mouse spleen and plated in the lower chamber while 4T1 breast cancer cells or its derivatives were plated into the upper chamber of a transwell. After 5 days of incubation, expression of iTregs markers (FoxP3, CD25 and CD4) were analyzed by flow cytometry. Data are expressed in % of FoxP3+ CD25+ over CD4+ T cells ± SD. *Experiment was done in triplicate.*

Activin-A knockdown was induced in 4T1 derivatives cells by adding doxycycline (2ug/mL) 4 days prior the experiment. Naïve CD4 T cells isolated from a healthy mouse spleen, stimulated with anti-CD3 (1mg/mL) and anti-CD28 (0.5mg/mL) were placed into the lower chamber of the transwell. Wild type (WT) $4T1^{WT}$ or its derivatives ($4T1^{shSCR}$ and $4T1^{shInhba}$) were then plated into the upper chamber of the hanger. After 5 days of incubation, the conversion of naïve CD4 T cells into iTregs ($CD4^+$ $CD25^+$ $FoxP3^+$ cells) was evaluated by flow-cytometry.

Culture of T cells in presence of $4T1^{WT}$ cells led to higher percentage of CD4 T cells differentiating into iTregs (9.58% ± 0.71 versus control 1.81% ± 0.04, **p=0.0042), which was significantly decreased (5.16% ± 0.47, p=0.0195 versus $4T1^{WT}$) by blocking activin-A with follistatin (FS), confirming our previous results hereby tumor-derived activin-A is partially responsible of the conversion of naïve CD4 T cells into iTregs. Additionally, co-culture of naïve T cells with 4T1 control scrambled shRNA-transduced cells ($4T1^{shSCR}$) w/o doxycycline resulted in similar percentage of iTregs, which was also decreased by inhibiting activin-A with FS, thus confirming that activin-A production by the **$4T1^{shSCR}$ is comparable to 4T1 WT cells, making them a suitable control for in vitro and in vivo experiments**. Most importantly, culture of naïve CD4 T cells in presence of $4T1^{shInhba}$ cells treated with doxycycline reduced iTregs conversion to 5.40%, a level comparable to coculture of 4T1 WT in the presence of FS. . Overall, these **results confirmed the successful knockdown of activin-A in 4T1 cells, and the role of activin-A in increasing conversion of naïve CD4+ T cells into iTregs.** .

Task 2.c*: *In vivo experiment with $4T1^{shSCR}$- and $4T1^{shInhba}$- tumor bearing mice.

To fully characterize our model, we first conducted in vivo experiment to determine

the impact of feeding animals with doxycycline on the growth of $4T1^{WT}$- and its derivatives. To that end, we injected 50,000 $4T1^{WT}$ or $4T1^{shSCR}$ subcutaneously (s.c.) in the flank of a BALB/c mouse. At day 10 after tumor cell injection, mice were fed doxycycline in drinking water at 200µg/mL or at 100µg/mL. Doxycycline did not impact $4T1^{WT}$ tumor growth at any concentration (Figure 7A). Without doxycycline, $4T1^{WT}$ and $4T1^{shSCR}$ showed similar tumor growth curves (Figure 7B). However, in presence of doxycycline, $4T1^{shSCR}$-tumor growth decreased at day 24 (Figure 7B) suggesting that

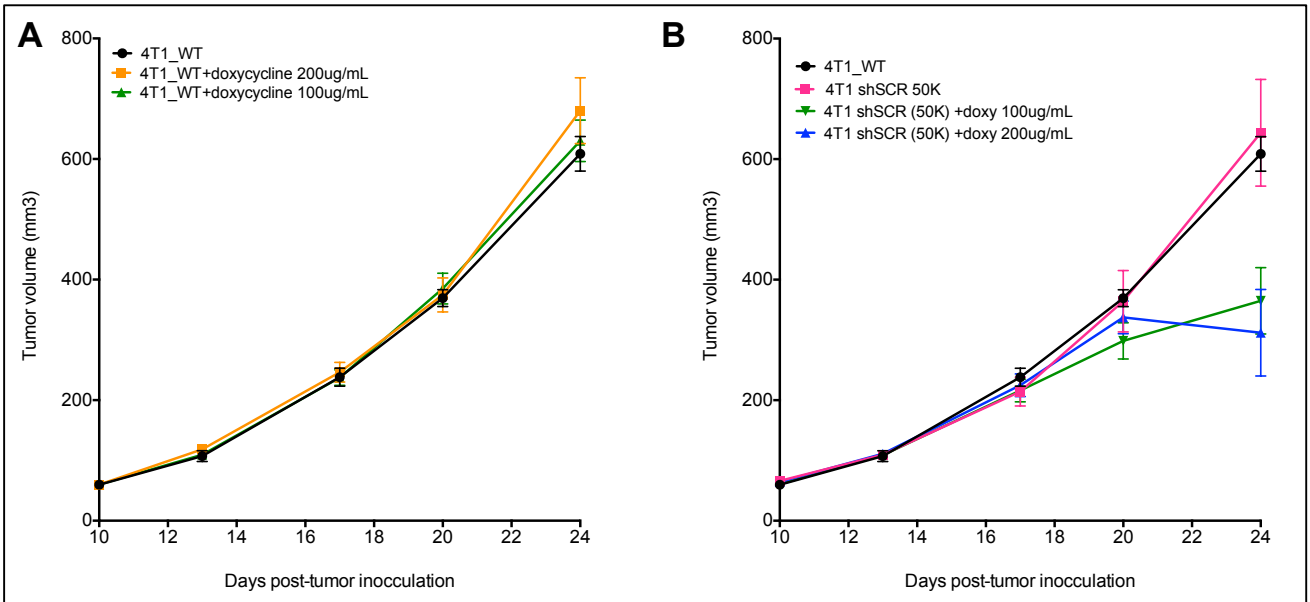

Figure 7: RFP induced by doxycycline reduced tumor growth in 4T1 control scrambled shRNA transduced cells. A-Syngeneic immunocompetent balb/c mice were inoculated with 50,000 (50K) $4T1^{WT}$ or its derivative $4T1^{shSCR}$ mammary carcinoma cells s.c on day 0. Animals were fed with doxycycline (200 µg/mL or 100µg/mL) starting day 8 (n=5 mice/group). (**A**) Tumor growth curves of $4T1^{WT}$ mice. (**B**) Tumor growth curves of $4T1^{shSCR.}$ mice. *Experiment was done in duplicate.*

induction of **RFP expression may be affecting $4T1^{shSCR}$ tumor growth**.

To overcome this effect, we increased the number of cells injected s.c. to 150,000 cells. As shown in Figure 8, injecting 3 times more tumor cells attenuated tumor growth delay previously observed. Therefore, **for future in vivo experiment we will inject 150,000 $4T1^{shSCR}$ or $4T1^{shInhba}$ cells and feed the animal with 100µg/mL of doxycycline**.

We then confirmed the inhibition of tumor-derived activin-A in vivo. To that end, we injected $4T1^{shSCR}$ or $4T1^{shInhba}$ cells into BALB/c mice and induced the *inhba* gene knockdown by feeding mice with doxycycline at 100ug/mL starting at day 8. At day 14 and day 25, tumors were collected, digested and stained for flowcytometry (n=3 per group). Viable CD45- RFP+ tumor cells were then sorted and RNA extracted for RT-qPCR to assess *Inhba* gene expression (Figure 9A and 9B). Results revealed that **tumor-derived activin-A is inhibited in vivo at both 14 and 25 days after tumor cells injection** (Figure 9C) indicating that sh*Inhba* construct is stable in vivo.

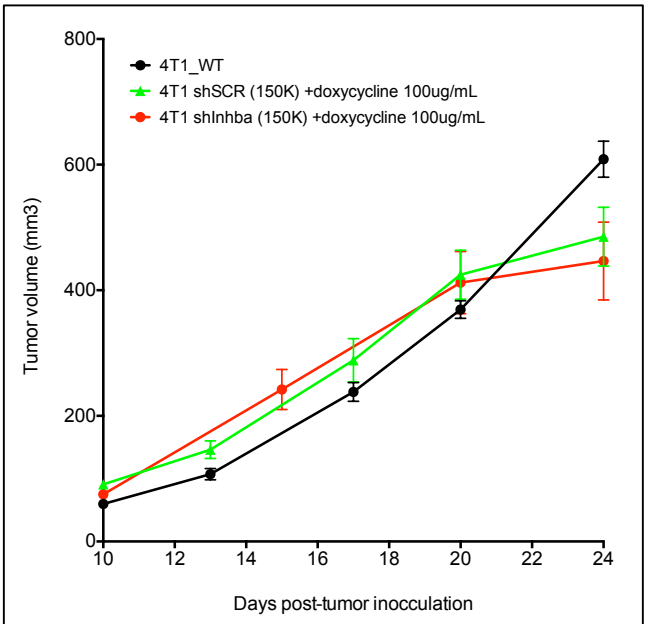

Figure 8: Tumor growth curves of $4T1^{WT}$, $4T1^{shSCR}$ and $4T1^{shInbha}$ tumor bearing mice w/o doxycyline. Syngeneic immunocompetent balb/c mice were inoculated with 150,000 (150K) $4T1^{WT}$ or its derivative $4T1^{shSCR}$ and $4T1^{shInhba}$ mammary carcinoma cells s.c on day 0. Animals were fed with doxycycline (100µg/mL) starting day 8 (n=5 mice/group).

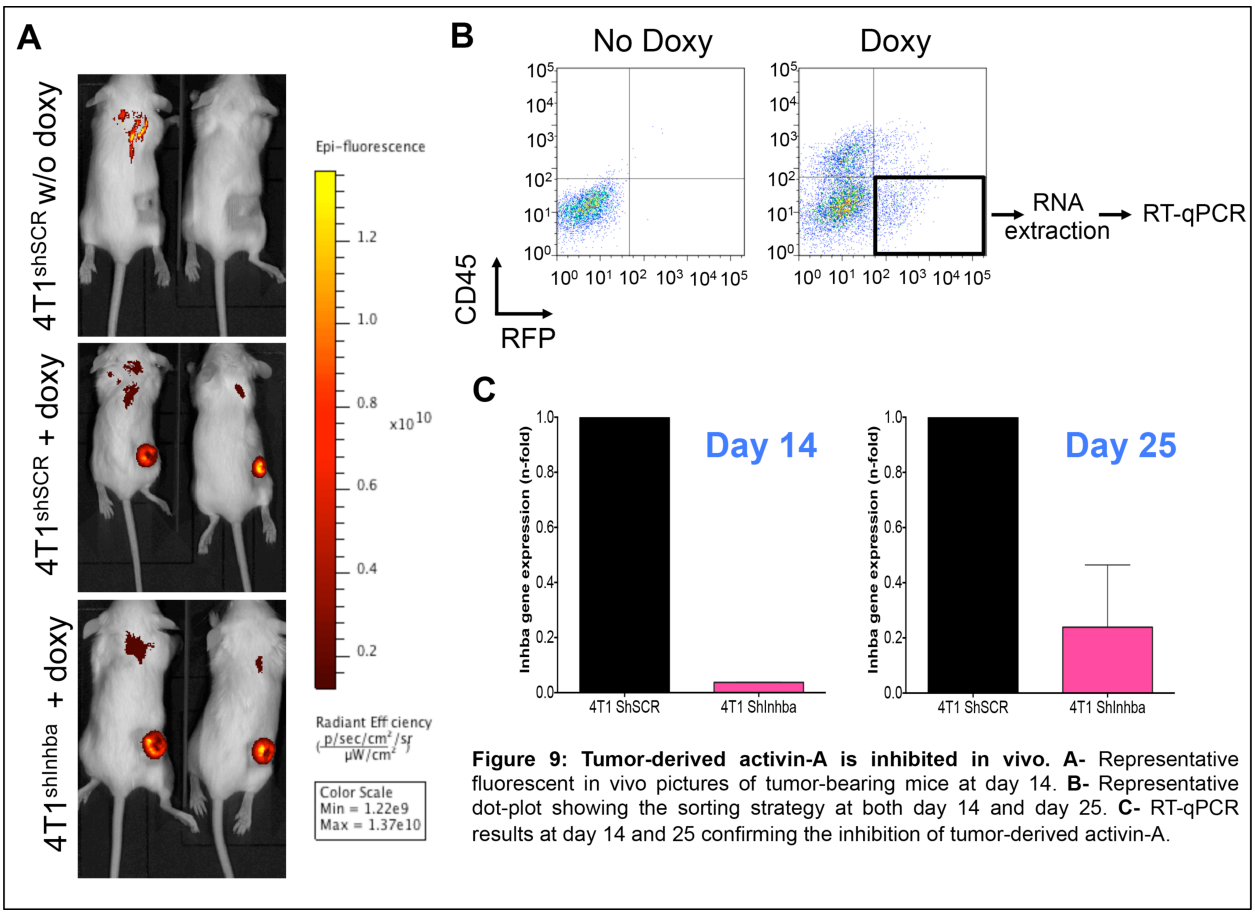

Figure 9: Tumor-derived activin-A is inhibited in vivo. A- Representative fluorescent in vivo pictures of tumor-bearing mice at day 14. **B-** Representative dot-plot showing the sorting strategy at both day 14 and day 25. **C-** RT-qPCR results at day 14 and 25 confirming the inhibition of tumor-derived activin-A.

3 Key Research Accomplishment

- The difficulty of generating stable 4T1 derivatives has been overcome
- pTRIPZ plasmid containing shInhba were prepared.
- Established $4T1^{shSCR}$ and $4T1^{shInhba}$ cells were obtained are shown to be stable in vitro and in vivo.
- Effective tumor-derived activin-A knockdown was demonstrated both in vitro and in vivo.
- The experimental parameters (tumorigenic incolum and doxycycline concentration) to study knockdown of activin-A in vivo were defined.

4 Reportable outcome

National Meeting and Presentations

- **2014 American Association of Cancer Research**

5-9 April 2014, San Diego, USA

Poster:

Inhibition of TGFβ as a strategy to convert the irradiated tumor into in situ individualized vaccine.

Vanpouille-Box C, Diamond J, Zavadil J, Babb J, Schaue D, Barcellos-Hoff MH, McBride WH, Formenti S and Demaria S.

- **2014 Radiation Research Society 60th annual meeting**

21-24 September 2014, Las Vegas, USA.

Marie Curie award oral presentation:

The type of radiation regimen modulates the ability of radiotherapy to generate an in situ tumor vaccine.

Vanpouille-Box C, Aryankalayil M, Pilones KA, Formenti S, Coleman N and Demaria S

- **2014 Society for Immunotherapy of Cancer 29th Annual Meeting**

6-9 November 2014, New Harbor, MA.

Poster*:*

Fractionated but not single dose radiation releases key signals of in situ tumor vaccination.

Vanpouille-Box C, Aryankalayil M, Pilones KA, Formenti S, Coleman N and Demaria S

Awards

- Marie Curie Award, Radiation Research Society, 2014.
- Scholar in training travel award, SITC, 2014

Publications

* Pilones KA, Vanpouille-Box C and Demaria S. *Combination of radiotherapy and immune checkpoint inhibitors*. **Semin Radiat Oncol**. 2015; 25(1):28-33.

* Vatner RE, Cooper BT, Vanpouille-Box C, Demaria S and Formenti S. *Combinations of immunotherapy and radiation in cancer therapy*. **Front. Oncol**. 2014; 4; 325.

* Demaria S, Pilones KA, Vanpouille-Box C, Golden E and Formenti SC. *The optimal partnership of radiation and immunotherapy: from pre-clinical studies to clinical translation*. **Rad Res**. 2014; Aug; 182(2):170-81.

Institutional meetings and conferences

- NYU Immunology Club
  Meets every Thursday 12PM

- NYU Cancer Institute Breast Biology Working Group
  Meets every 3rd Wednesday of every month

- NYU Molecular Oncology and Tumor Immunology Works-in-Progress
  Meets every Tuesday 5PM

- NYU Patho-Biology Works-in-Progress
  Meets every Tuesday 6PM

- NYU Immunology and Inflammation Works-in-Progress
  Meets every Wednesday 5:30PM

Mentoring

- Julie Diamond

Rotating student August 2012-December 2012.
Joined Sandra Demaria Lab after her rotation.

Collaboration

- Mary Helen Barcellos-Hoff, PhD.

Department of radiation oncology, NYU School of Medicine

We are closely collaborating with the lab of Pr Barcellos-Hoff on mechanisms of TGF-beta inhibition in cancer. Our collaboration has contributed significantly to our understanding of the immunosuppressive mechanisms within the tumor microenvironment by TGF-beta superfamily members.

Conclusion

Collectively, I succeeded in generating 4T1 derivatives to study the effect of tumor-derived activin-A in vivo. Obtained data confirm my previous in vitro results from year 1 hereby tumor-derived activin-A is partially responsible for the conversion of naïve CD4 T cells into iTregs. Therefore, I expect that blocking activin-A should decrease immunosuppression in vivo. These experiments, as described in the approved statement of work, will comprise much of the work to be done in the third year.

In the past year, I have actively participated in departmental Works-in-Progress seminars and attended to NYU immunology club presentations as well as NYU Cancer Institute Breast Biology Working Group sessions, which have enriched my knowledge in cutting edge research in breast cancer. I have had the great opportunity to meet with leaders in the field of breast cancer immunology at recent meetings, and will continue to foster a collaborative relationship with them in the years to come. I continue to work closely with my mentor, Dr Sandra Demaria, who I meet with every week to discuss results and plan experiments. She continues to be an invaluable resource to my training as a future breast cancer scientist.

5 Appendices

APPENDIX 1

Abstract 633: *Inhibition of TGFβ as a strategy to convert the irradiated tumor into in situ individualized vaccine.* Vanpouille-Box C, Diamond J, Zavadil J, Babb J, Schaue D, Barcellos-Hoff MH, McBride WH, Formenti S and Demaria S.

APPENDIX 2

(AL03) *The type of radiation regimen modulates the ability of radiotherapy to generate an in situ tumor vaccine.* Vanpouille-Box C, Aryankalayil M, Pilones KA, Formenti S, Coleman N and Demaria S.

APPENDIX 3

Vanpouille-Box C, Aryankalayil M, Pilones K, Formenti S, Coleman NC and Demaria S. *Fractionated but not single dose radiation releases key signals of in situ tumor vaccination*.
Journal for ImmunoTherapy of Cancer 2014, 2(Suppl 3): P164.

APPENDIX 4

Pilones KA, Vanpouille-Box C and Demaria S. *Combination of radiotherapy and immune checkpoint inhibitors*. Semin Radiat Oncol. 2015; 25(1):28-33.

APPENDIX 5

Vatner RE, Cooper BT, Vanpouille-Box C, Demaria S and Formenti S. *Combinations of immunotherapy and radiation in cancer therapy*. Front. Oncol. 2014; 4; 325.

APPENDIX 6

Demaria S, Pilones KA, Vanpouille-Box C, Golden E and Formenti SC. *The optimal partnership of radiation and immunotherapy: from pre-clinical studies to clinical translation*. **Rad Res**. 2014; Aug; 182(2):170-81.

POSTER PRESENTATION – 2014 AACR annual meeting.

Abstract 633: Inhibition of TGFβ as a strategy to convert the irradiated tumor into in situ individualized vaccine.

Claire I. Vanpouille-Box[1], Julie M. Diamond[1], Jiri Zavadil[1], James Babb[2], Dörthe Schaue[3], Mary Helen Barcellos-Hoff[4], William H. McBride[3], Silvia C. Formenti[4], and Sandra Demaria[1]

[1]NYU School of Medicine, Department of Pathology, New York, NY; [2]NYU School of Medicine, Department of Radiology, New York, NY; [3]UCLA, Department of Radiation Oncology, Los Angeles, CA; [4]NYU School of Medicine, Department of Radiation Oncology, New York, NY.

Accumulating data support the concept that ionizing radiation therapy (RT) has the potential to convert the tumor into an in situ, individualized vaccine; however this potential is rarely realized by RT alone. Transforming growth factor β (TGFβ) is an immunosuppressive cytokine that is activated by RT and inhibits the antigen-presenting function of dendritic cells and the differentiation of effector CD8+ T cells. Here we tested the hypothesis that TGFβ hinders the ability of RT to promote anti-tumor immunity. Development of tumor-specific immunity was examined in two pre-clinical models of metastatic breast cancer and analyzed in patients with metastatic breast cancer treated with local radiotherapy and the TGFβ-neutralizing antibody Fresolimumab.

Mice bearing established 4T1 and TSA mouse mammary carcinomas treated with pan-isoform specific TGFβ neutralizing antibody, 1D11, showed significantly improved control of the irradiated tumor and non-irradiated metastases, but no effect in the absence of RT. Notably, whole tumor transcriptional analysis demonstrated the selective upregulation of genes associated with immune-mediated rejection only in tumors of mice treated with RT+TGFβ blockade. Mice treated with RT+TGFβ blockade exhibited cross-priming of CD8+ T cells producing IFNγ in response to three tumor-specific antigens in tumor-draining lymph nodes, which was not evident for single modality treatment. Likewise, HLA-A2.1+ metastatic breast cancer patients (n=8) enrolled in NCT01401062 trial of local RT and fresolimumab were examined for CD8+ T cells specific for the tumor antigen survivin by tetramer staining. Three patients developed increased frequencies of survivin-specific CD8+ T cells in the blood during treatment, while two patients negative at baseline became positive.

Analysis of the immune infiltrate in mouse tumors showed a significant increase in CD4+ and CD8+ T cells only in mice treated with the combination of RT+TGFβ blockade. Depletion of CD4+ or CD8+ T cells abrogated the therapeutic benefit of RT+TGFβ blockade.

These data identify TGFβ as a master inhibitor of the ability of RT to generate an in situ tumor vaccine, which supports testing inhibition of TGFβ during radiotherapy to promote therapeutically effective anti-tumor immunity.

Supported by DOD BCRP Multi-Team Award BC100481.

MARIE CURIE AWARD LECTURE – 60th RRS annual Meeting.

AL03 - The type of radiation regimen modulates the ability of radiotherapy to generate an in situ tumor vaccine.

Claire I. Vanpouille-Box, PhD[1]; Molykutty J. Aryankalayil[2]; Karsten A. Pilones, MD, PhD[1]; Silvia C. Formenti[1]; Norman Coleman[2]; and Sandra Demaria[1],
New York University School of Medicine, New York, NY[1] and National Cancer Center Institute, Bethesda, MD[2].

Local radiotherapy (RT) promotes cross-priming of anti-tumor T cells generating an individualized in situ vaccine. Induction of therapeutically effective anti-tumor responses is modulated by the balance between pro-inflammatory and immunosuppressive signals pre-existing in the tumor microenvironment and generated by RT. We have shown that the dose and fractionation employed play a key role in determining if this balance is shifted in favor of anti-tumor immunity. In two tumor models, generation of an in situ vaccine synergistic with anti-CTLA-4 treatment was achieved by irradiation of the tumor with 3 fractions of 8 Gy (8Gyx3) but not by a single 20 Gy dose (20Gyx1) (Dewan et al., Clin Cancer Res 2009).
To understand the mechanisms underlying the different outcome obtained with fractionated versus single dose regimens, TSA tumors growing in syngeneic immunocompetent BALB/c mice were harvested at 4, 24 and 48 hrs post-RT for analysis of isolated RNA by microarray or infiltrating immune cells by flow cytometry. Expression of key immune genes in TSA cells irradiated in vitro was assessed by qPCR.
Tumors irradiated in vivo showed the rapid induction of hundreds of immune response genes by 8Gyx3 but not 20Gyx1, with a dominant type I interferon (IFN) response at 4 and 24 hours, which was confirmed by qRT-PCR. CD8α+ dendritic cells (DC), which are the subset of DC cross-presenting tumor cell-derived antigens, showed a significant upregulation of activation markers CD86, CD40 and CD70 at 48 hours following 8Gyx3 but not 20 Gyx1. Importantly, the in vitro setting (devoided of an immune infiltrate) demonstrated expression of IFNβ and downstream immune genes, including chemokines CXCL9, CXCL10 and CXCL11 by TSA cells irradiated with 8Gyx3 but not 20Gyx1.
Data indicate that fractionated RT can mimic, at least in part, a viral infection and activate canonical defense pathways in neoplastic epithelial cells with induction of type-I IFN. In vivo this leads to activation of DC cross-presenting tumor antigens. This suggests that fractionated-RT generates the key “ingredients” of an in situ tumor vaccine. Further studies to identify the molecular mechanisms of RT-induced tumor vaccination and their modulation by different RT regimens are critical to the rationale design of clinical trials testing RT combinations with immunotherapy.

Vanpouille-Box et al. Journal for ImmunoTherapy of Cancer 2014, 2(Suppl 3):P164
http://www.immunotherapyofcancer.org/content/2/S3/P164

Journal for ImmunoTherapy of Cancer

POSTER PRESENTATION Open Access

Fractionated but not single dose radiation releases key signals of in situ tumor vaccination

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The balance between pro-inflammatory and immunosuppressive signals in the tumor microenvironment dictates the responsiveness of the immune system. Local radiotherapy (RT) has the potential to switch this balance in favor of anti-tumor immunity by promoting cross-priming of anti-tumor T cells thus generating an individualized *in situ* vaccine. We have previously shown that the dose and fractionation employed modulate RT ability to synergize with immunotherapy. Indeed, in two tumor models, generation of an *in situ* vaccine synergistic with anti-CTLA-4 treatment was achieved by irradiation of the tumor with 3 fractions of 8 Gy (8Gyx3) but not by a single 20 Gy dose (20Gyx1) (Dewan *et al*., Clin Cancer Res 2009).

To understand the mechanisms underlying the different outcome obtained with fractionated (3x8Gy) versus single dose (20Gyx1) RT, TSA tumors growing in syngeneic immunocompetent BALB/c mice were harvested at 4, 24 and 48 hrs post-RT for analysis of purified RNA by microarray for gene expression or infiltrating immune cells by flow cytometry. Expression of key immune genes in TSA cells irradiated *in vitro* was assessed by qPCR.

Over 100 immune response genes were differentially expressed in irradiated tumors by 8Gyx3 but not 20Gyx1, with a dominant type I interferon (IFN) response at 4 and 24 hours, which was confirmed by qRT-PCR. CD8a+ dendritic cells (DC), which are the subset of DC cross-presenting tumor cell-derived antigens, showed a significant upregulation of activation markers CD86, CD40 and CD70 at 48 hours following 8Gyx3 but not 20 Gyx1. Importantly, the *in vitro* setting (devoid of an immune infiltrate) demonstrated expression of IFNb and downstream immune genes, including chemokines CXCL9, CXCL10 and CXCL11 by TSA cells irradiated with 8Gyx3 but not 20Gyx1.

Data indicate that fractionated RT can mimic, at least in part, a viral infection and activate canonical defense pathways in neoplastic epithelial cells with induction of type-I IFN. *In vivo* this leads to activation of DC cross-presenting tumor antigens, suggesting that the quality of fractionated-RT generates the key "ingredients" of an *in situ* tumor vaccine. Further studies to identify the molecular mechanisms of RT-induced tumor vaccination and their modulation by different RT regimens are critical to the rational design of clinical trials testing RT combinations with immunotherapy.

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Combination of Radiotherapy and Immune Checkpoint Inhibitors

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The ability of ionizing radiation to cause cell death and inflammatory reactions has been known since the beginning of its therapeutic use in oncology. However, only recently this property of radiation has attracted the attention of immunologists seeking to induce or improve antitumor immunity. As immune checkpoint inhibitors are becoming mainstream cancer treatments, radiation oncologists have begun to observe unexpected out-of-the-field (abscopal) responses in patients receiving radiation therapy during immunotherapy. These unexpected responses were predicted by experimental work in preclinical tumor models and have clear biological bases. Accumulating experimental evidence that radiation induces an immunogenic cell death and promotes recruitment and function of T cells within the tumor microenvironment supports the hypothesis that radiation can convert the tumor into an in situ individualized vaccine. This property of radiation is key to its synergy with immune checkpoint inhibitors, antibodies targeting inhibitory receptors on T cells such as cytotoxic T lymphocyte antigen-4 and programmed death-1. By removing the obstacles hindering the activation and function of antitumor T cells, these agents benefit patients with pre-existing antitumor immunity but are ineffective in patients lacking these spontaneous responses. Radiation induces antitumor T cells complementing the activity of immune checkpoint inhibitors.
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Introduction

Antigens recognized by T cells in tumors include differentiation antigens, overexpressed antigens, cancer-testis, and mutated tumor neoantigens.[1] Only antigens in the last category are truly tumor specific, whereas the other antigens are expressed at low levels or in a restricted fashion in normal adult tissues or during development and are often referred to as "tumor-associated" antigens. Because tumor-associated antigens are shared between multiple tumors and patients, they have been the focus of most vaccination strategies. However, clinical responses have been limited even when antigen-reactive T cells were successfully induced by the vaccine.[2] In part, this may be because such self-antigens can only elicit weak responses as strongly reactive T cells are usually deleted during ontogeny. In addition, because of their intrinsic genomic instability, cancer cells can escape cytotoxic T lymphocyte (CTL) recognition by mutation or downregulation of the antigens, just as they almost inevitably develop resistance to targeted therapeutics. In fact, the process of immunoediting of antigens expressed by tumors occurs spontaneously during cancer development.[3] Tumors arising in immunocompetent hosts lose the most immunogenic antigens under the pressure of the immune system to escape immune control.[4]

Importantly, the same process that allows immune escape also generates a plethora of mutated neoantigens that are more immunogenic than differentiation or overexpressed antigens.[5,6] Tumors with the highest degree of genomic instability often also have a more prominent lymphocytic infiltrate.[7,8]

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They escape immune control by recruiting immunosuppressive and regulatory host cells, producing immunosuppressive cytokines and other mediators, and by expressing surface ligands that inhibit CTL action.[9] Perhaps not surprisingly, tumors with an immune-active microenvironment are more likely to respond to immunotherapy agents that target key immunosuppressive pathways.[10] The remarkable clinical responses to treatment with anti–CTL antigen-4 (CTLA-4) or anti–programmed death (PD)-1 antibodies or both observed in metastatic melanoma, a tumor with a high mutational burden, illustrates this concept.[11-13]

However, responses to immune checkpoint inhibitors are seen only in a fraction of patients with melanoma and other cancers. Efforts to identify combination treatment that can convert nonresponders into responders who could enjoy the long-term benefits of immunotherapy are ongoing.[14] As part of this effort, radiotherapy is being tested in combination with the Food and Drug Administration–approved anti–CTLA-4 antibody ipilimumab (Yervoy, Bristol Meyers-Squibb, New York, NY) in at least a dozen trials (www.clinicaltrials.gov). Herein, we review the preclinical data that provide a rationale for testing radiotherapy as potentially an ideal partner for immune checkpoint inhibitors and discuss the initial clinical observations that support a synergy between radiation and anti–CTLA-4 therapy.

Evidence That Local Radiotherapy Can Generate an In Situ Tumor Vaccine

Ionizing radiation causes damage to multiple biomolecules by direct energy deposition or by generation of free radicals, leading to cell death when the damage cannot be repaired.[15] Tumor cell death induced by radiation is usually preceded by cell stress and, depending on the degree of alteration of survival and apoptosis pathways and of cell cycle regulatory mechanisms, can occur via different pathways.[16] However, it is likely that at least a portion of the cancer cells within a tumor will die an "immunogenic cell death" when radiation is used at therapeutic doses.[17] The latter has been defined as a cell death that is associated with the generation of specific molecular signals that are sensed by antigen-presenting cells (APC) and stimulate their maturation and ability to cross-present tumor-derived antigens to T cells.[18,19] In addition, production of type I interferon (IFN) has been shown to be required for optimal activation of antitumor T cells by radiation.[20] Thus, tumor cells that die following radiotherapy can activate canonical pathways of response to infections and potentially elicit powerful antitumor innate and adaptive immune responses.

The changes induced by radiation in the tumor microenvironment can contribute to fueling this process.[21] Radiation induces chemokines that attract effector T cells to the tumor and vascular adhesion molecules that facilitate T-cell infiltration.[22-24] Downstream of homing, tumor cells that survive after radiation upregulate a number of cell surface molecules, including major histocompatibility Class I, Fas (CD95), ICAM-1, and NKG2D ligands becoming "optimized" targets for CTLs.[25-27] This process, which has been named "immunogenic modulation,"[28] may play a role not only in regression of the irradiated tumor but also in amplifying and strengthening adaptive antitumor immunity. The ongoing process of killing of tumor cells by CTLs sustains release of more tumor antigens and possibly promotes antigenic spread, that is, activation of a broader T-cell repertoire. In support of this hypothesis, Gulley et al[29] reported evidence of antigenic spread in some patients with prostate cancer who were treated with the combination of vaccination and standard local radiotherapy.

Despite the multiple proimmunogenic effects of radiation, the contribution of antitumor immunity to the response of an irradiated tumor remains to be demonstrated in patients, and systemic antitumor responses following local radiotherapy are rare. Such "out-of-the-field" or abscopal responses have been occasionally observed, and reports can be found in the medical literature of the past 60 years.[30] Increasing use of hypofractionated and ablative radiation, thought to be more proimmunogenic,[31] has not resulted in a noticeable increase in abscopal responses. Recently, with the introduction of immune checkpoint inhibitors as a standard treatment for metastatic melanoma, several cases of abscopal responses have been noted in patients receiving palliative radiation therapy during ipilimumab treatment.[32-34] Such responses were predicted by our data demonstrating a synergy of radiation with anti–CTLA-4 antibody in mice tumor models.[35,36] Although the reproducibility of abscopal responses is being tested in clinical trials, and the mechanisms involved may differ in patients and mice, it is reasonable to hypothesize that the ability of radiotherapy to generate an in situ tumor vaccine might generate the immune-active tumor microenvironment required for response to immune checkpoint inhibitors.

Immune Checkpoint Receptors

Cytotoxic T Lymphocyte Antigen-4

CTLA-4 (CD152) is a master regulator of T-cell activation that plays a key role in maintaining tolerance to self-antigens, as demonstrated by the development of lymphoproliferative disease with massive T-cell infiltration of multiple organs in CTLA-4 knockout mice.[37,38] However, in the immunosuppressive microenvironment of cancer, CTLA-4 becomes an obstacle to the activation and function of antitumor T cells. T-cell activation is initiated when T-cell receptors (TCR) bind to antigenic peptide presented in the context of major histocompatibility molecules. Binding of CD28 coreceptor to costimulatory molecules CD80 (B7-1) and CD86 (B7-2) on the surface of APCs provides the required second signal to stimulate T-cell proliferation and cytokine production.[39] CTLA-4 is rapidly recruited to the immune synapse after TCR triggering and competes with CD28 for binding to CD80 and CD86.[40] Because it has higher affinity for costimulatory molecules than CD28, when the latter are present in limiting number, CTLA-4 prevails[41] (Fig.). In addition to precluding the proproliferative signals delivered via CD28, CTLA-4 also recruits phosphatases that dephosphorylate key effector molecules blunting TCR signaling.[42,43]

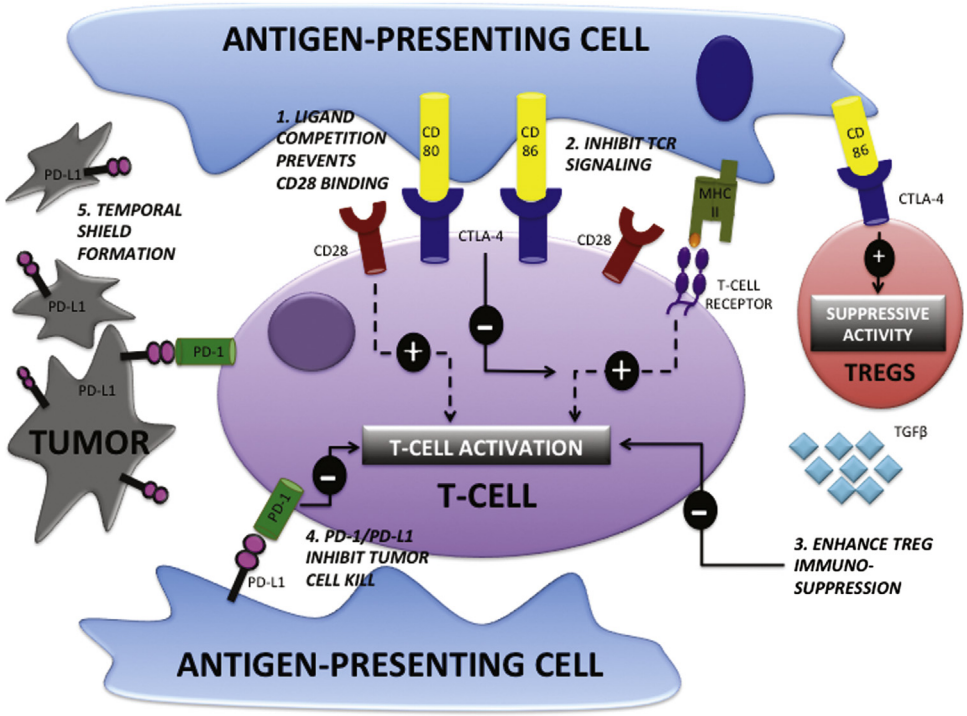

Figure Mechanisms of CTLA-4– and PD-1–mediated regulation of T-cell activation. (1) CTLA-4 molecules can act as high-affinity competitors for B7-family ligands (CD80 and CD86) on the surface of APCs and prevent CD28 from delivering activation signals to T-cells. (2) CTLA-4 ligation can also recruit phosphatases, which directly inhibit activation signals from the T-cell receptors. (3) Extrinsic control of immune activation occurs through Tregs, which express CTLA-4 molecules constitutively. Binding of CTLA-4 to B7 ligands promotes Treg suppressive function and stimulates production of immunosuppressive cytokines such as TGF-β. (4) Activated T cells also express inhibitory PD-1 receptors, which bind to their ligands on APCs. (5) Proinflammatory IFNγ secreted by activated T cells can also induce tumor cells to express PD-L1, which forms a temporal "shield" inhibiting T-cell–mediated cytotoxicity. TGF-β, transforming growth factor beta. (Color version of figure is available online.)

CTLA-4 also regulates T-cell function via other nonmutually exclusive mechanisms. For example, CTLA-4 engagement regulates integrin-dependent motility and prevents T cells from forming long-term interactions with APCs or target cells, which are necessary to sustain T-cell activation and cytotoxic activity.[27,44] Importantly, CTLA-4 is expressed constitutively on $CD4^+$ regulatory T cells (Tregs) and mediates signals promoting production of the highly suppressive cytokine transforming growth factor beta.[45] Because of their relatively high expression of CTLA-4, Tregs also compromise APC function by reducing the availability of B7 ligands that can engage CD28 molecules required to activate antitumor T cells.[46,47] The molecular mechanism of this effect involves CTLA-4–mediated capture of B7 ligands by transendocytosis. The internalization and subsequent degradation of these key ligands by Tregs deprives APCs of their ability to provide costimulation required for T-cell activation.[48] In cancer, a combination of these mechanisms contributes to the profound immunosuppression within the tumor microenvironment. Persistent tumor antigen exposure drives exhaustion of T cells marked by higher expression of CTLA-4 and other immune checkpoint receptors. This together with impaired APC costimulation and enhanced Treg immunosuppression contributes to a significantly muted antitumor immune response.

Programmed Death-1

PD-1 (CD279) is another inhibitory receptor expressed by T cells on activation that plays a critical role in maintenance of peripheral tolerance. PD-1 knockout mice with an autoimmune-prone background show accelerated development of autoimmune diseases.[49] In contrast to CTLA-4, however, PD-1 has a more restricted role and is thought to be mainly involved in limiting damage to normal tissue during inflammatory responses.[50] Belonging to the B7 family, 2 PD-1 ligands have been identified: PD-L1 (aka B7-H1 or CD274) and PD-L2 (aka B7-DC or CD273). The affinity of PD-L2 for PD-1 is 3-fold higher than PD-L1, but its expression is restricted largely to myeloid cells.[51] In contrast, PD-L1 is broadly expressed on hematopoietic and nonhematopoietic cells. PD-L1 and PD-L2 expression is regulated by proinflammatory cytokines. For instance, type-1 and type-2 IFN, IL-10, and TNFα induce PD-L1 expression on T cells, B cells, endothelial cells, and epithelial cells,[52,53] whereas IL-4 and granulocyte-macrophages colony-stimulating factor (GM-CSF) stimulate the expression of PD-L2 on dendritic cells.[54]

Upregulation of PD-L1 is common in tumors and has been correlated with progression and poor prognosis.[55-57] Because PD-L1 expression is induced by IFNγ produced by T cells and has been seen in tumors in association with an immune infiltrate, it is thought to be a mechanism of "induced

resistance" whereby tumor cells escape T-cell attack.[58] In fact, PD-L1 expression by cancer cells was shown to increase the rate of apoptosis of tumor-specific CTLs, an effect partially reversed by anti–PD-L1 mAb.[55] Additionally, PD-L1 can transmit an antiapoptotic signal to tumor cells through its intracellular domain, thus shielding tumor cells from CTL-mediated killing[59-61] (Fig.).

Combination of Local Radiotherapy With Checkpoint Receptor Blockade

CTLA-4 blockade

In a seminal study, antibody-mediated blockade of CTLA-4 was shown to induce effective antitumor immunity in mice.[62] The therapeutic effect, however, was limited to relatively immunogenic tumors. Less immunogenic tumors, including the B16 mouse melanoma model, required the addition of a vaccine to anti–CTLA-4 antibody to achieve tumor rejection[63]. For patients with metastatic melanoma, anti–CTLA-4 mAbs have shown clinical benefits as single agents.[11] Although the percentage of patients responding is limited to 10%-15%, responses are long-lasting demonstrating the advantage of targeting the immune system over targeting the cancer cells themselves.[14] In an attempt to improve the response rates, anti–CTLA-4 mAb was tested in combination with a peptide vaccine derived from the Glycoprotein-100 tumor-associated antigen. Results failed to show any synergy.[11] There is an important difference between the formulation of the vaccines that were able to show synergistic effects with anti–CTLA-4 mAb in otherwise nonresponsive mouse tumors and the Glycoprotein-100 vaccine used in patients. Effective vaccines were made of irradiated autologous tumor cells that had been transduced to express GM-CSF.[64] The latter have been shown to contain mutated tumor neoantigens that could prime stronger and broader antitumor immune responses.[65]

We were the first to show that local irradiation of a tumor growing in mice could mimic the effects of vaccination with GM-CSF–producing autologous tumor cells and could convert a tumor resistant to anti–CTLA-4 mAb into a tumor that was sensitive to it.[35] This synergy of local radiotherapy with anti–CTLA-4 mAb was seen in different tumor models. The treated mice developed abscopal responses mediated by activation of powerful antitumor T cells, some directed to an endogenous tumor antigen.[36] This suggests that local radiation was indeed generating an in situ tumor vaccine. Induction of a chemokine that enhanced CTLs recruitment to the irradiated tumor and of NKG2D ligands on the tumor cells, required for formation of an immune synapse between tumor cells and CTLs, contributed to the synergy of radiation with anti–CTLA-4 mAb.[22,27] Data support a model whereby "waves" of tumor cell killing by T cells primed by the initial radiation-elicited antigen release boost the immune response. This process can eventually achieve systemic tumor control.

Multiple reports in patients with melanoma unresponsive to ipilimumab who developed abscopal responses following irradiation of a single metastasis[32-34] suggest that radiotherapy's generation of an in situ tumor vaccine is a probable event in humans. Strikingly, we have recently shown that such abscopal responses can be seen in tumor types that do not respond to anti–CTLA-4 treatment. Irradiation of a single liver metastasis in a patient with lung cancer with widespread disease treated with ipilimumab led to a complete and durable response.[66] Results of several ongoing clinical trials testing the combination of radiotherapy with anti–CTLA-4 treatment will provide required evidence of its benefits.

Blockade of PD-1 or PDL-1 Pathway

Antibodies targeting PD-L1 or PD-1 have been shown to promote CTL expansion[67] and tumor regression in many mouse tumor models.[68-71] This demonstrates the importance of this pathway in tumor escape from immune-mediated control. These findings have been successfully translated to patients with objective responses (partial or complete) reported in 28% with melanomas, 27% with renal cell carcinoma, and 18% of patients with non–small cell lung cancer.[12,72] Although these results are impressive and testing is ongoing in other tumor types, the proportion of responders remains a minority. Combinations that can recruit more patients into responding are being actively investigated.

Because it is thought that response to anti–PD-1 or anti–PD-L1 treatment is limited to patients with pre-existing antitumor T-cell responses that are unleashed by these antibodies, strategies to induce such responses are potential candidates for testing in combination with anti–PD-1 or anti–PD-L1 treatment. Local radiotherapy is being investigated in several laboratories, including our own, and initial preclinical studies have shown promising results. In a mouse glioma model, the combination of radiation with anti–PD-1 therapy significantly increased median survival, with some mice exhibiting cure and development of antitumor memory responses.[73] In another study, superior tumor control of implanted breast and colorectal carcinomas was demonstrated when anti–PD-L1 mAbs were used in combination with radiotherapy.[74] Importantly, an abscopal response was demonstrated only in mice given combined therapy.

Conclusions

The well-orchestrated expression of negative regulatory molecules in immune cells prevents unrestricted T-cell activation that can potentially lead to immunopathology. In cancer, these immune checkpoint pathways are disregulated and tend to be overexpressed, preventing tumor rejection. Preclinical and clinical data have demonstrated that even in advanced cancer resistant to other treatment, these inhibitory receptors can be successfully targeted therapeutically. Importantly, radiotherapy is emerging as an optimal partner for immune checkpoint inhibitors because of its ability to induce response in patients who are otherwise nonresponsive.

If radiation is confirmed to be a "universal" sensitizer of tumors to immune checkpoint inhibitors in clinical trials, its

application will be easy to implement and widespread. Although the mechanisms involved in the synergy of radiation with anti–CTLA-4 and anti–PD-1 or anti–PD-L1 treatment need to be further studied in patients, there are several other immune checkpoint receptors that are under active investigation as therapeutic targets in cancer. For example, preclinical studies have provided compelling evidence that lymphocyte activation gene-3 and T-cell immunoglobulin-3 may be excellent targets for cancer immunotherapy.[75,76] Thus, radiotherapy is assuming a new role as an immune adjuvant in a new era of cancer immunotherapy.

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Combinations of immunotherapy and radiation in cancer therapy

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The immune system has the ability to recognize and specifically reject tumors, and tumors only become clinically apparent once they have evaded immune destruction by creating an immunosuppressive tumor microenvironment. Radiotherapy (RT) can cause immunogenic tumor cell death resulting in cross-priming of tumor-specific T-cells, acting as an *in situ* tumor vaccine; however, RT alone rarely induces effective anti-tumor immunity resulting in systemic tumor rejection. Immunotherapy can complement RT to help overcome tumor-induced immune suppression, as demonstrated in pre-clinical tumor models. Here, we provide the rationale for combinations of different immunotherapies and RT, and review the pre-clinical and emerging clinical evidence for these combinations in the treatment of cancer.

Keywords: ionizing radiation, radiotherapy, immunotherapy, tumor immunity, clinical trials, microenvironment, abscopal effect

INTRODUCTION

This review aims at providing the reader with both the rationale for and the emerging information regarding pre-clinical and clinical testing of combinations of different immunotherapies and radiotherapy (RT). We will first provide a summary of the main mechanisms cancer harnesses to evade the control of the immune system, then we will describe some of the available evidence for the effects of ionizing radiation on the immune system. We will then focus on examples of clinical studies built on this background and share some of the preliminary results that are emerging. Hopefully, this review will succeed at motivating more pre-clinical and clinical research in the novel field of combined radiation and immunity.

CANCER'S CROSS-TALK WITH THE HOST'S IMMUNE SYSTEM

The adaptive human immune system can specifically recognize up to 10^{12} unique antigens, allowing T-cells to discriminate between transformed cells and normal self (1–3). There is evidence in animal models, and indirect evidence in human beings, that a competent immune system can selectively eliminate cancer cells and protect against the development of tumors (4–9). This evidence is corroborated by the increased incidence of malignancies in immune-suppressed individuals such as AIDS patients and recipients of allograft transplants (10–13). This raises the question: if the immune system can eliminate cancers, how do cancers develop in the context of a competent immune system?

Schreiber's modification of the immunosurveillance hypothesis addresses this question, proposing that tumors must undergo three processes before they become clinically apparent: elimination, equilibrium, and escape (14, 15). In the elimination phase, transformed cells are recognized by cognate $CD8^+$ cytotoxic T-lymphocytes (CTLs) and are immediately eliminated through cytotoxic mechanisms such as Fas/Fas–ligand interactions and granzyme/perforin mediated killing. This process continues until some transformed cells evolve means to evade killing by CTLs. It is hypothesized that a phase of equilibrium forms between newly transformed cell clones and those effectively eliminated by CTLs (16). Eventually, cancer cells able to evade elimination by CTLs acquire more mutations, and develop unregulated growth, invasion, and metastases. Each of these steps is associated with active evasion of the immune system.

MECHANISMS FOR IMMUNE EVASION

Tumors have the entire genome at their disposal for modulating and evading the anti-tumor-immune response, and their escape tends to be multi-pronged (**Figure 1**). One simple method of escape utilized by tumors and viruses alike, is down-regulation or inactivation of the cellular machinery responsible for MHC class I (MHC-I) antigen processing and presentation (17–20). If tumor peptide antigens are not presented by MHC-I, CTLs cannot recognize and eliminate transformed cells, although MHC down-regulation does make tumors more susceptible to NK cell cytotoxicity (21, 22).

Another common mechanism for disrupting the immune response is through interference with CTL priming, primarily through modification of the intratumoral infiltrate of dendritic cells (DCs) (3–5, 8, 9, 23). Intratumoral DCs often have an immature or regulatory phenotype that results in the presentation of tumor antigens without co-stimulation, resulting in cross-tolerance and anergy of T-cells (24–27). The importance of this mechanism in tumor-immune escape is highlighted by the close temporal correlation of antigen-specific tolerance of both $CD4^+$ and $CD8^+$ tumor-specific T-cells with the outgrowth of experimental tumors (6, 7, 14, 15). Additionally, regulatory DCs (regDCs) can have direct effects on tumor-immune escape, as the transfer of regDCs into tumor-bearing mice is sufficient to promote tumor growth and metastasis (16, 28).

Perhaps the most common and effective means of interfering with anti-tumor immunity is by blocking the effector function of CTLs through various mechanisms. Tumors foster the

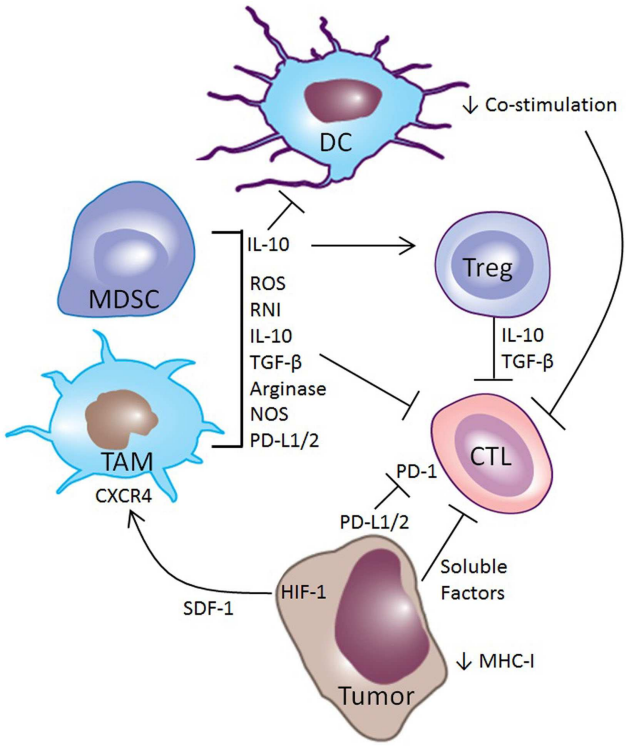

FIGURE 1 | Mechanisms of immune suppression in the tumor microenvironment. Tumors utilize multiple mechanisms for evading the immune system. Tumor cells can down-regulate expression of MHC-I, making them poor targets for CTL mediated killing. Along with myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), they can express PD-L1 and PD-L2, which inhibit CTL function through the PD-1 receptor. Tumors make other soluble factors that also inhibit CTLs. Hypoxia in tumors induces HIF-1, driving the production of SDF-1, which acts as a chemokine to attract MDSCs and TAMs to the tumor microenvironment through the receptor CXCR4. These MDSCs and TAMs secrete cytokines such as IL-10 that promote a regulatory phenotype among intratumoral DCs, induce Tregs, and directly inhibit CTLs. Other myeloid-derived factors that inhibit CTL activity include TGF-β, reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), and arginase and nitric oxide synthase (NOS), which are enzymes that deplete l-arginine, an important metabolite for CTL function.

development of an immunosuppressive microenvironment by recruiting Tregs and myeloid elements – primarily tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) – that make TGF-β and IL-10 (29–32). These anti-inflammatory cytokines blunt anti-tumor immunity by inhibiting the cytolytic activity of CTLs. Furthermore, TAMs and MDSCs modify the metabolic milieu of the tumor microenvironment by producing arginase and nitric oxide that deplete L-arginine, an essential nutrient for T-cell function (33–35). These suppressive myeloid cells also generate reactive oxygen and nitrogen species that modify the chemokine and antigen receptors on CTLs both in the lymphoid organs and in the tumor, impairing their ability to home to tumors and kill tumor cells (36).

The tumor vasculature plays an important role in tumor-induced immune dysregulation. Tumors often outgrow their vasculature, and abnormal tumor angiogenesis results in tumor ischemia and hypoxia, which initiates recruitment of immunosuppressive myeloid cells (37). Low oxygen tension in tumors promotes an increase in hypoxia inducible factor-1 (HIF-1), which stimulates the production of stromal-derived factor-1 (SDF-1). SDF-1 acts as a chemokine, recruiting myeloid-derived cells through the chemokine receptor CXCR4 (38, 39). Furthermore, as the gatekeeper between the blood and the tumor microenvironment, the tumor vasculature plays a direct role in modulating anti-tumor immunity. Recruitment of immunosuppressive TAMs, MDSCs, regDCs, and Tregs, as well as anti-tumor CTLs, requires active engagement of the vascular endothelium in the tumor (40). While chemokine gradients attract these immune cells to the tumor, extravasation requires the expression of selectins and integrins, such as E-selectin, ICAM-1, and VCAM-1 for rolling, activation, arrest, and transmigration (41). Endothelial cells can even selectively recruit subsets of leukocytes, such as Tregs, which has been described in hepatocellular carcinoma and pancreatic cancer (42, 43). In addition to these effects, tumor cells and vascular endothelium can directly dysregulate or kill effector CTLs through engagement of the Programed Death-1 (PD-1) receptor by expressing PD-1 ligand (44–47). Current immunotherapy strategies target these mechanisms in the attempt to overcome immune escape of cancer and recover immune-rejection (48).

EFFECT OF RADIOTHERAPY ON CANCER IMMUNE RESPONSE

Radiotherapy, while traditionally used for its direct cytocidal effect on cancer cells, also has immunomodulatory properties and can be harnessed to potentiate an immune response (49, 50) (**Figure 2**). Ionizing radiation causes immunogenic cell death of cancer cells, modulates antigen presentation by cancer cells, and most importantly alters the microenvironment within the irradiated field (51–54). Lymphocytes are exquisitely sensitive to ionizing radiation, and the direct effect of RT on tumor-infiltrating lymphocytes is generally cytocidal (55). This results in temporary selective ablation of immune cells within the irradiated target, depleting CTLs and NK cells directed against the tumor as well as Tregs that suppress local anti-tumor immunity. The relative importance of the effect of RT on these populations remains unclear but it is evident that the damaging effects of this physical insult are sensed by the immune system, with systemic implications.

Radiation-induced immunogenic cell death is characterized by the release of tumor antigens in the context of endogenous adjuvants that facilitates priming of anti-tumor CTLs (56). Important components of immunogenic cell death include translocation of calreticulin (CRT) to the tumor cell membrane and release of ATP and other endogenous adjuvants such as HMGB1 (57), uric acid (58), and heat-shock proteins (HSPs) (59, 60). These endogenous adjuvants act through the toll-like receptors (TLRs) to facilitate DC maturation (61–63). The role of TLRs in the mammalian immune system was first described as pattern recognition receptors that respond to pathogen associated molecular patterns (PAMPs) such as endotoxin from bacteria and double stranded RNA from viruses (64). However, there is growing evidence that the TLRs have a broader function by mediating the response to danger associated molecular patterns (DAMPs) (65). DAMPs are a larger class of molecules including PAMPs in addition to

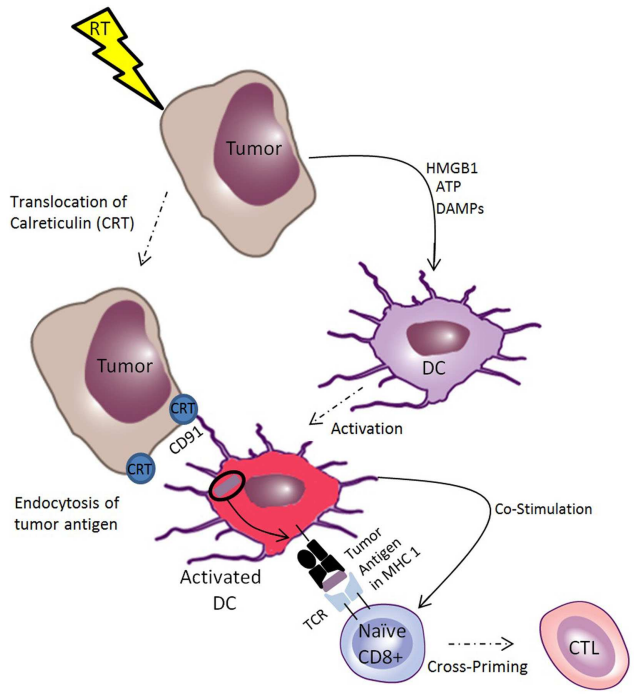

FIGURE 2 | Ionizing radiation induces immunogenic cell death of tumors, which facilitates cross-priming of CTLs. Ionizing radiation induces translocation of calreticulin (CRT) to the tumor cell membrane, which acts as an "eat me" signal to dendritic cells (DCs), facilitating receptor mediated endocytosis through CD91. This makes tumor antigens available for cross-presentation on MHC-I for priming of tumor-specific T-cells. Radiotherapy also induces the release of danger associated molecular patterns (DAMPs), such as ATP and HMGB-1, which are endogenous immune adjuvants that stimulate DC activation, inducing DCs to provide co-stimulatory signals to naïve T-cells, facilitating cross-priming of CTLs. Together, these processes constitute immunogenic cell death of tumor cells.

endogenous, evolutionarily conserved intracellular molecules that are released upon necrotic cell death. By linking the innate and adaptive immune system by activating antigen-presenting cells, release of DAMPs is a key aspect of immunogenic cell death mediated by RT.

Another key component of the pro-immunogenic effect of RT is the facilitation of tumor antigen uptake by DCs and cross-presentation on MHC-I (66). In fact, radiation induces MHC-I in both tumors and normal tissue (67, 68). By enhancing presentation of antigens released by its cytocidal effect, RT potentiates cross-priming of tumor-specific CTLs in the lymph nodes. Exogenous antigens can access the cross-presentation pathway by a variety of means but the most important for anti-tumor immunity is the uptake of cell-associated antigens mediated by the translocation of CRT from the endoplasmic reticulum of tumor cells to the cell surface. Ionizing radiation causes CRT to translocate to the tumor cell surface where it acts as an "eat me" signal to macrophages and DCs, which internalize CRT expressing tumor cells (69). This process is mediated by the common HSP receptor CD91, and is a necessary part of anthracycline and radiation-induced immunogenic cell death (70–72). Radiation induces the translocation of CRT on the tumor cell surface along with the release of the DAMPs HMGB1 and ATP. These signals have been shown to be necessary and sufficient in a model of radiation-induced anti-tumor immunity (73, 74).

There is evidence from both human beings and mice that tumor-associated antigens are cross-presented by DCs after RT, and this results in cross-priming of tumor-specific CTLs. By experimental necessity, much of this evidence comes from murine tumor lines transfected to express model antigens, which allow for measurement of specific CTL responses against known peptide epitopes. A single fraction of 20 Gy of ionizing radiation results in cross-presentation of an epitope from the SIY model antigen, demonstrated by an elegant set of experiments performed *in vivo* using a melanoma model (75). In a different melanoma model, both a single 15 Gy fraction of RT and fractionated RT resulted in cross-priming of CTLs detected in the tumor and tumor draining lymph nodes, with fractionated treatment resulting in a smaller degree of cross-priming (76). Other investigators have used this model to study the effect of dose and fractionation on cross-priming, and have found the number of CTLs generated correlates with the dose of radiation, but after fractionated treatment all doses of RT resulted in about the same number of primed CTLs (77). This RT induced cross-priming is dependent on TLR-4 signaling in the host (57). These findings are consistent with evidence from patients with prostate cancer who developed prostate specific CTLs after RT and vaccination with a poxviral vaccine encoding prostate specific antigen (PSA) (78).

Immunogenic cell death alone may not be sufficient to mediate a robust anti-tumor-immune response since the resident DCs within tumors maintain tolerance (3). Intratumoral injection of exogenous DCs have been used as an immune therapy for cancer, and RT has been shown to stimulate an effective anti-tumor CTL response among patients treated with this method (79–82). In some experimental systems, RT overcame the suppressive effect of tumor resident DCs by recruiting new myeloid-derived DCs that have not been exposed to the regulatory effects of the tumor microenvironment. Tumor irradiation recruits these monocyte derived DCs (mDCs) to tumors after treatment with a single large fraction of 25 Gy (83). In summary, RT induces multiple intracellular adhesion molecules (ICAMs), chemokines, and cytokines that mediate naïve DC recruitment and may at least in part subvert the immune-tolerant microenvironment characteristic of established tumors (84–86).

Furthermore, RT facilitates the recruitment of effector T-cells to tumors through the induction of chemokines. Chemokines are known to be important for the recruitment of leukocytes to tumors as part of anti-tumor immunity (87, 88). However, tumors with their immunosuppressive milieu tend to produce chemokines that recruit Tregs and other suppressive elements (89, 90). Without effective chemotaxis, lymphocytes primed against tumor antigens cannot home to tumors and carry out their effector function. CXCL16 is a chemokine that has been identified as a prognostic factor that correlates with improved survival and increased numbers of tumor-infiltrating lymphocytes in colorectal cancer and renal cell carcinoma (91–93). RT induces CXCL16 production in the 4T1 mouse breast cancer model, which mediates T-cell

recruitment to tumors through the CXCR6 receptor on T-cells (94). Radiation also has effects on the tumor vascular endothelium, inducing cell adhesion molecules that further promote recruitment of anti-tumor CTLs (95). Although it does not explain the systemic immune effects of RT, chemotaxis induced by RT may partially account for the direct effects of RT on tumor control.

THE ABSCOPAL EFFECT OF RADIOTHERAPY

The effects of ionizing radiation on the anti-tumor-immune response support the hypothesis that the immune system is responsible for the abscopal effect of RT. Originally described by Mole, the abscopal (from the Latin *ab* and the Greek *scopus*, away from the target) effect of radiation therapy is a phenomenon by which a primary tumor is irradiated and a response is seen at distant metastatic sites outside of the path of the radiation (96–102). Our group has generated pre-clinical evidence that it is mediated by the immune system (103–105). Even the "in field" effects of radiation have been shown to be dependent on the immune system, as $CD8^+$ T cells and type I interferon are required for tumor regression after radiation therapy, since their depletion abrogates tumor control after RT (75, 83, 106–108).

Despite the observation that radiation induces effects sensed by the immune system and modulates the immune response to tumors, abscopal responses are rarely seen in clinical practice. Although there is evidence that radiation therapy alone is sufficient to provide the necessary signals for cross-priming of CTLs against tumor antigens, this adjuvant effect of radiation appears to be relatively weak. However, the rare radiation-induced systemic abscopal response can be facilitated when additional immune manipulation is added. RT primes new anti-tumor CTLs but these CTLs are usually unable to overcome the suppressive effect of the tumor microenvironment at distant untreated metastatic sites. This is the rationale for combining systemic immunotherapies with RT.

ANTI-IMMUNOGENIC EFFECTS OF RT

It must be noted that RT has anti-immunogenic effects in addition to the pro-immunogenic effects described above. There are reports that RT can impair DC function, including cross-priming (109, 110). Additionally, RT can contribute to the immune-suppressive tumor microenvironment by recruiting MDSCs and TAMs (76, 111–113). Tumor-infiltrating Tregs are also enriched after RT (77, 114). The relative importance of these immunosuppressive effects of RT remains unclear and it is likely to be model-dependent, since there are contrasting reports of RT resulting in a shift toward a macrophage mediated pro-immunogenic microenvironment (115).

COMBINATIONS OF RADIOTHERAPY AND IMMUNOTHERAPY IN THE CLINIC

There have been a number of efforts recently to combine immunotherapy with RT to augment the anti-tumor-immune effects of RT. Abscopal responses to RT alone are extremely rare, suggesting that combinations with immunotherapy may be required to sustain the pro-immunogenic effects of radiation. Similarly, only a small proportion of cancer patients derive objective benefit from currently available immunotherapies. One strategy to increase both the likelihood and duration of systemic anti-tumor immunity in response to immunotherapy is to add RT as an adjunct to bolster the immune response. When combined with RT, immunotherapeutic approaches can be broadly separated into (1) the promotion of cross-priming of tumor-specific CTLs, (2) the stimulation of immune effector function of CTLs primed by RT, and (3) neutralization of the immunosuppressive effects of the tumor microenvironment. Essentially, all current clinical approaches fall into the first two categories, with the third category primarily in the pre-clinical stage.

PROMOTION OF CROSS-PRIMING OF TUMOR-SPECIFIC CTLs

GROWTH FACTORS TO FACILITATE RECRUITMENT OF DCs

The use of growth factors to recruit DCs from the bone marrow to the irradiated tumor was based on the very first animal model of the abscopal effect. In this model, syngeneic breast cancer cells were implanted subcutaneously into the bilateral flanks of Balb/c mice. Once the tumors grew into palpable nodules the tumor on one side was treated with RT and systemic fms-like tyrosine kinase-3 (flt-3) ligand was given concomitantly to recruit DCs from the bone marrow. The combination of RT and flt-3 ligand inhibited growth of both the irradiated tumor and the contralateral untreated tumor. This abscopal effect in a tumor nodule outside of the radiation field was demonstrated to be tumor-specific and was not observed when the experiment was repeated in nude mice, which lack T-cells, suggesting an immune-mediated mechanism (104). Due to the lack of clinical availability of flt-3 ligand, GM-CSF – another DC growth factor – was substituted when these pre-clinical studies were translated into a proof of principle pilot study at our institution for patients with metastatic solid tumors. GM-CSF increases the percentage of DCs and promotes their maturation; facilitating cross-presentation of newly released antigens after cancer cell death is achieved within the irradiated tumor. In this study, one measureable metastatic lesion was treated to a dose of 35 Gy in 10 fractions, and starting on day seven (after 1 week of radiation) GM-CSF ($125\,\mu g/m^2$) was administered subcutaneously every day for 14 days. Abscopal response was defined as a measurable response in any of the measurable lesions outside the radiation field, assessed by PET-CT. Results of this trial were reported at the American Society for Therapeutic Radiation Oncology (ASTRO) annual meeting in 2012, and a manuscript describing the long-term outcome of the treated patients is in preparation. A weakness of this study was the lack of immune-monitoring available for these patients.

INTRATUMORAL INJECTION OF AUTOLOGOUS DCs

A more direct, albeit labor intensive, method for delivering DCs to the site of tumor antigen release after RT is by direct injection. For this therapy, autologous DCs are generated from mononuclear cells isolated by leukapheresis from peripheral blood by culturing these *in vitro* in the presence of cytokines and growth factors (GM-CSF). These DCs are then reintroduced directly into the irradiated tumor by injection. In one study utilizing this method, five $HLA\text{-}A2^+$ patients with high-risk prostate cancer were treated with androgen suppression, 45 Gy of external beam RT and intraprostatic DC injections after fractions 5, 15, and 25. Serial prostate biopsies before and during treatment showed apoptosis of tumor cells and an increase in tumor-infiltrating $CD8^+$ T-cells, as well as an increase in prostate specific $CD8^+$ T-cells in the peripheral blood (116). This approach has also been used neoadjuvantly

in patients with high-risk soft tissue sarcoma. Seventeen patients were treated to 50.4 Gy in 28 fractions with intratumoral injection of 10^7 DCs, three times during treatment and once near surgery to assess for cell migration. Nine patients (53%) developed tumor-specific immune responses, which lasted up to 42 weeks with 12 of 17 patients (71%) free of progression at 1 year (117). There is one small completed randomized trial using this approach, investigating radiation therapy with and without intratumoral DC injection. Preliminary results reported 5/14 patients exhibiting an enhanced T-lymphocyte response in the experimental arm versus 2/6 in the control arm (ClinicalTrials.gov identifier: NCT01347034).

Intratumoral injection of DCs has also been used in patients with refractory hepatoma in combination with 8 Gy, single-fraction RT. All 14 patients in this study tolerated the treatment, while half of the patients had a minor or partial response clinically, and 8 patients developed an AFP specific immune response (118). There is an ongoing proof of principle trial studying the combination of RT with intratumoral DC injection in patients with malignant melanoma (ClinicalTrials.gov identifier: NCT01973322). Another recently completed phase I/II study examined the combination of intratumoral DC injection with gemcitabine and hypofractionated stereotactic body radiation therapy (SBRT) in the setting of unresectable pancreatic cancer, but results are pending (ClinicalTrials.gov identifier: NCT00547144).

DC ACTIVATION USING TLR AGONISTS

Another approach to improving T-cell cross-priming in response to RT is to activate intratumoral DCs using TLR agonists, thus improving the ability of DCs to present tumor antigens released by RT and to provide co-stimulation to naïve T-cells. This results in more robust priming and effector function of tumor-specific CTLs. Many different TLR ligands, both natural and synthetic, have been utilized in conjunction with RT to boost anti-tumor immunity. PSK, a protein-bound polysaccharide derived from the fungus *Basidiomycete coriolus versicolor* has been shown to activate NK cells and DCs through TLR2, leading to its use in conjunction with chemoRT in locally advanced rectal cancer (119, 120). Thirty patients were treated with the oral antimetabolite radiosensitizing chemotherapy S-1 in combination with neo-adjuvant radiation (20 Gy in 10 fractions) followed by radical surgery with intra-operative electron therapy (15 Gy). Patients were randomized between PSK given three times a day at a dose of 3 g/day or placebo during the neo-adjuvant external beam portion of the treatment. There was a significant increase in the percentage of NK cells in the peripheral blood and an increase in number of CTLs in the rectal mucosa, as well as a decrease in the immunosuppressive acidic protein level in the serum of patients treated with PSK (121). A suspension of heat killed *Mycobacterium obuense*, called IMM–101, also contains TLR2 agonists, which has been shown to be safe and well tolerated in human beings (122). The combination of IMM–101 and single-fraction linear accelerator based stereotactic radiosurgery is currently being tested in a single arm, phase II study in patients with previously treated metastatic colorectal cancer (ClinicalTrials.gov identifier: NCT01539824). Similarly, a hot water extract from bacillus tuberculosis called Z-100, containing polysaccharides such as arabinomannan and mannin, has immunomodulatory properties (123). This was tested in a Japanese phase III, randomized trial in patients with stage IIB – IVA cervical cancer in conjunction with standard of care chemoRT with cisplatinum. A total of 249 patients were randomized to biweekly subcutaneous injections of Z-100 or placebo and concurrent RT. Z-100 demonstrated a trend toward increased overall survival ($p = 0.07$), although the statistical power of this study was less than anticipated because survival rates were higher than expected for both arms (124). There is also an ongoing trial of the TLR4 agonist glucopyranosyl lipid A in combination with five to six fractions of RT in patients with metastatic sarcoma, (ClinicalTrials.gov identifier: NCT02180698).

TLR3 is the receptor for poly-ICLC, a synthetic double stranded RNA shown to increase the antibody response to antigen and augment the activation of NK cells, macrophages, and T-cells (125, 126). The North American Brain Tumor Consortium conducted a single-arm phase II trial of patients with recurrent anaplastic glioma, testing 20 mcg/kg poly-ICLC administered three times weekly by intramuscular injection in combination with 200 cGy daily RT to the recurrent brain tumor to a total dose of 60 Gy followed by poly-ICLC for up to 1 year, or until tumor progression. Thirty eligible patients demonstrated a 1-year overall survival of 69%, which compares favorably to the group treated with RT alone (127).

TLR9 agonists have also been the target of investigation of combined immunoradiotherapy. Brody et al. injected the CpG DNA PF-3512676 into the tumors of 15 patients with low-grade B-cell lymphoma treated concurrently with low-dose RT, resulting in a 27% response rate (128). The success of this approach led to its application in mycosis fungoides in a phase I/II study that demonstrated a 33% response rate and a trend toward a reduction of $CD25^+$ T-cells (primarily Tregs) and dermal DCs in the clinical responders (129).

Imiquimod is a synthetic imidazoquinoline, which specifically activates TLR7, expressed by both plasmacytoid DCs and $CD11c^+$ myeloid-derived DCs (130). In pre-clinical models, we have shown that RT in combination with imiquimod significantly improves survival of tumor-bearing mice treated with either modality alone, and based on these results we initiated an ongoing phase I/II study of imiquimod and RT for patients with breast cancer metastatic to the skin or recurrent on the chest wall (ClinicalTrials.gov identifier: NCT01421017) (131, 132). Imiquimod is also being used in a pilot study in combination with concurrent radiation in an attempt to improve outcomes in diffuse intrinsic pontine glioma, a pediatric brain tumor with a poor prognosis (ClinicalTrials.gov identifier: NCT01400672).

CYTOTOXIC GENE THERAPY

Cytotoxic gene therapy delivered *in situ* is a different tactic for improving the radiation-induced anti-tumor-immune response. This method employs intratumoral injection of recombinant viruses carrying genes that induce tumor-specific cell death, which complements the immunogenic cell death induced by RT. Cancer gene therapy using herpes simplex virus thymidine kinase (HSV-tk) in combination with gancyclovir, acyclovir, or valacyclovir to induce tumor cell death and anti-tumor immunity in combination with RT has been used with moderate success in patients with prostate cancer. After completing a phase I trial to establish

safety in 18 men, this approach was tested in 33 men with intermediate and high-risk features in combination with definitive RT and anti-hormonal therapy (133). With a median follow-up of 26 months, mean percentages of DR^+CD8^+ T cells were increased at all time-points up to 8 months with DR^+CD4^+ T cells increased later and sustained longer until 12 months (134). The same group is conducting three parallel trials as salvage treatment in patients who progress after RT, as neo-adjuvant treatment prior to radical prostatectomy, and in combination with definitive RT. The addition of RT significantly increased both $CD4^+$ and $CD8^+$ T-cells in peripheral blood when compared to the methods lacking combined RT, adding support to combined modality therapy (135). This led to the initiation of a phase III multi-center randomized trial that will be very important in establishing the efficacy of this approach (ClinicalTrials.gov identifier: NCT01436968). We will also learn of the potential activity of this approach in patients with malignant glioma (ClinicalTrials.gov identifier: NCT00589875, NCT00751270) and pediatric brain tumors (ClinicalTrials.gov identifier: NCT00634231), and using a similar approach in pancreatic cancer (ClinicalTrials.gov identifier: NCT00638612), with completion and reporting of ongoing trials.

VACCINES

Therapeutic cancer vaccines promote anti-tumor immunity by stimulating T-cell priming against tumor antigens, or peptide antigens thought to be specific or cross-reactive with tumors. This is another method that acts in parallel with RT for inducing anti-tumor immunity, and is often given with exogenous immunostimulatory adjuvants that promote cross-priming of T-cells against the vaccine antigen as well as antigens released by RT. Pre-clinical studies support the synergistic effect of therapeutic vaccination with RT. For example, a combination of 8 Gy delivered with a recombinant vaccinia-carcinoembryonic antigen vaccine (CEA) resulted in rejection of CEA expressing colon cancer, an effect that was not observed when the treatments were given individually (136). Human studies mimic these results.

One powerful effect of tumor vaccines is the ability to jump-start the anti-tumor-immune response to both vaccination and RT, inducing a phenomenon known as an "antigen cascade" or "epitope spreading" (137). Initially discovered in models of autoimmune disease, and more recently described after administration of peptide-based cancer vaccines, epitope spreading describes the generation of T-cells specific for distinct and non-cross reactive tumor antigens after vaccination against known antigens (138). This phenomenon was particularly well characterized after peptide-based vaccination for prostate cancer that was administered concurrently with standard definitive RT. In this phase II trial, 30 men with clinically localized prostate cancer were randomized 2:1 to receive vaccine plus prostate directed RT or RT alone. The vaccine consisted of a recombinant vaccinia viral vector coding for PSA and the co-stimulatory molecule B7.1, and was administered concurrently by subcutaneous injection with GM-CSF and low-dose IL-2, followed by monthly booster vaccination with recombinant fowlpox-PSA. Eight patients had extensive analysis of their PBMCs for tumor-specific T-cell responses, and six of these eight patients developed T-cells specific for multiple tumor-associated antigens that were not included in the vaccine, such as PAP, MUC-1, PSMA, and PSCA (78). This suggests vaccination against a single tumor antigen along with RT can spark an antigenic cascade that results in an immune response against many endogenous tumor antigens. Most vaccine trials do not specifically incorporate RT for its immunogenic properties, and will not be described here.

STIMULATION OF IMMUNE EFFECTOR FUNCTION OF CTLs

CYTOKINES TO BOLSTER IMMUNE EFFECTOR FUNCTION

One approach to improving the efficacy of tumor-specific T-cells induced by RT is to bolster the effector function of these T-cells and other leukocytes through the use of cytokines. Interferons are a group of proteins that are secreted by DCs, lymphocytes, macrophages, fibroblasts, and other leukocytes, that increase the activity of immune effector cells and make cancer cells into better immune targets by increasing antigen processing and presentation (139). The combination of interferon alpha and chemoradiation provides a survival advantage over chemoradiation alone in early studies of patients with completely resected pancreatic cancer (140). Unfortunately, the treatment is toxic, with 95% of patients developing grade 3 or higher toxicity. This has led to the premature closure of ACOSOG Z05031, a randomized trial assessing a similar treatment strategy, and until now, other randomized trials have failed to show a benefit to combined adjuvant chemoradiation with immunotherapy for resected pancreatic cancer (141, 142).

Similar toxicity was observed when tumor necrosis factor-alpha (TNF-α) in combination with radiation was tested for locally advanced and metastatic tumors. This phase I trial resulted in a 23% patient withdrawal rate due to major toxicity (143). In an attempt to improve the tolerability of TNF-α therapy, TNFerade was developed; a replication deficient adenovector that expresses human TNF-α under the control of the radiation-inducible Egr-1 promoter. This was first tested in human beings in conjunction with radiation in a phase I trial involving 36 patients with solid tumors, of whom 70% had an objective response with no dose-limiting toxicities (144). Phase I and II studies were subsequently conducted in soft tissue sarcoma, rectal cancer, pancreatic cancer, esophageal cancer, and recurrent head and neck cancer (145–149). The promising results in the locally advanced pancreatic cancer setting led to a multi-institutional, phase III randomized trial of concurrent fluorouracil and RT with or without TNFerade. Three hundred and four patients were randomized 2:1 in favor of TNFerade treatment. Lack of benefit in progression-free or overall survival dampened the optimism for this therapeutic approach in this tumor setting (150).

Interleukin 2 (IL-2) is a cytokine that is necessary for the growth, proliferation, and differentiation of T-cells to become antigen-specific $CD4^+$ and $CD8^+$ T-cells. IL-2 has been used with meager success for both melanoma and renal cell carcinoma (151, 152). Pre-clinical studies demonstrated increased cytokine release (153, 154) and up-regulated expression of MHC-I (68), B7.1 (155), and Fas/CD95 (156, 157) with the addition of radiation. This inspired a phase I study combining IL-2 and SBRT for patients with metastatic renal cell carcinoma and melanoma in which 2/3 of the patients demonstrated a response, and immune-monitoring looking at cryopreserved PMBCs showed a significantly greater

frequency of proliferating $CD4^+$ T cells with an early activated effector memory phenotype (158).

A phase II study is ongoing, looking at the combination of IL-2 and SBRT in patients with metastatic renal cell carcinoma to assess for both a local and systemic response with the rationale that large fractions of radiation (8–20 Gy) in combination with IL-2 will increase antigen presentation and immune stimulation (ClinicalTrials.gov identifier: NCT01896271). A similar strategy is being employed by the Dutch in the setting of oligometastases in an ongoing phase I trial (ClinicalTrials.gov identifier: NCT02086721). In an attempt to decrease the toxicity of IL-2 treatment, there is an industry sponsored phase II trial combining SBRT with MSB0010445, a modified IL-2 cytokine bound to a monoclonal antibody specific for DNA, which localizes the treatment to necrotic cells (ClinicalTrials.gov identifier: NCT01973608).

ENHANCEMENT OF T-CELL CO-STIMULATION

Co-stimulation refers to the activating signals delivered to T-cells – along with antigen-specific stimulation through engagement of the T-cell receptor – that are required for effective priming and anti-tumor effector function (159). More generally, this is an important tool used by the immune system to prevent autoimmunity by ensuring the presence of DAMPs at the time of T-cell priming. The use of TLR agonists, described above, indirectly enhances co-stimulation and priming of tumor-specific T-cells; however, agonists of the co-stimulatory receptors can be utilized to directly promote co-stimulation and improved activation and effector function of anti-tumor T-cells. There are two general families of co-stimulatory molecules, the B7/CD28 immunoglobulin family and the TNF/TNFR family (160). The stimulatory B7-family members include CD80 (B7-1) and CD86 (B7-2), which stimulate T-cells through CD28, and CD275 (ICOS-L), which acts through CD278 (ICOS) (161). The TNF/TNFR family includes CD154 (CD40L), CD252 (OX40L), CD70, and CD137L (4-1BBL), which signal through CD40L, CD134 (OX40), CD27, and CD137 (4-1BB), respectively.

Many of these co-stimulatory molecules and pathways are already targets for anti-cancer therapy, but there is more limited experience combining them with RT. Monoclonal antibody agonists of CD40 improve the efficacy of DC based immunotherapy (162), and are showing promise in combination with standard chemotherapy (163–165). Antibody agonists to 4-1BB are also showing promise as immunotherapy, especially in combination with other immunotherapies (166, 167). For example, overall survival was improved in a murine glioma model when radiation was combined with a 4-1BB agonist and blockade of cytotoxic T-lymphocyte antigen-4 (CTLA-4). As predicted, treatment with the triple therapy resulted in a higher density of $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes when compared to RT or either immunotherapeutic agent alone (168). Furthermore, 4-1BB activation augments the effects of RT in the murine M109 lung cancer and EMT6 breast cancer models, in which a single dose up to 15 Gy or fractionated RT up to 20 Gy slowed tumor growth to a significantly greater extent in combination with an antagonist antibody to 4-1BB (169).

OX40 is one of the more powerful co-stimulatory receptors expressed on activated T-cells, and signaling through OX40 is capable of breaking tolerance (170). Signaling through OX40 by OX40 ligand or monoclonal antibody agonists stimulates T-cells to proliferate, produce cytokines, and improve their effector function (171, 172). In a pre-clinical model of lung cancer transfected with an experimental antigen, a combination of a monoclonal antibody OX40 agonist with a single fraction of 20 Gy resulted in improved tumor response and increased antigen-specific $CD8^+$ T-cells that were not observed with either treatment alone (173). There is an ongoing phase Ib clinical trial testing the effect of cyclophosphamide, RT, and an antibody agonist of OX40 in patients with metastatic prostate cancer (ClinicalTrials.gov identifier: NCT01303705). In a way, this is actually two types of immunotherapy combined with RT. Although cyclophosphamide is a conventional chemotherapy, when given in low doses it tends to selectively deplete Tregs over effector T-cells, thus removing a barrier from the anti-tumor-immune response (174, 175). This effect of cyclophosphamide was first discovered 40 years ago, and is only now being utilized in clinical trials to modulate anti-tumor immunity (176). Cyclophosphamide (300 mg/m^2) is administered intravenously on day 1, followed by a single 8 Gy dose of RT on day 4 treating up to three osseous metastases along with the OX40 agonist treatment, which is repeated every 2 days for a total of three doses. There is a similar study of patients with metastatic breast cancer combining OX40 agonist treatment with SBRT utilizing doses ranging from a single fraction of 15 Gy up to two fractions of 20 Gy (ClinicalTrials.gov identifier: NCT01642290). Safety and immune correlates are the primary outcome measures of these trials, but early results have not yet been reported.

CHECKPOINT BLOCKADE TO BOLSTER CTL EFFECTOR FUNCTION

A reciprocal approach that is also effective for boosting effector T-cell function is to block the immune checkpoints that counteract endogenous co-stimulation of activated T-cells (177). Immune checkpoints are a collection of endogenous mechanisms for preventing unchecked T-cell activation and runaway immune responses after effector T-cells have neutralized an infectious or neoplastic threat. Checkpoint receptors, including CTLA-4 and PD-1, are up-regulated on activated T-cells and transmit inhibitory signals, which suppress T-cell proliferation and function (159). For example, in addition to the co-stimulatory receptor CD28, activated T-cells also express CTLA-4, which directly competes for binding to the co-stimulatory ligands CD80 and CD86 (178). CTLA-4 acts as a natural checkpoint to prevent indefinite activation of T-cells, and inhibition of this immune checkpoint with a monoclonal antibody antagonist to CTLA-4 shifts the balance of co-stimulation toward increased proliferation and function of activated T-cells, including tumor-specific CTLs.

There is extensive data, both pre-clinical and from patients, demonstrating the effectiveness of CTLA-4 blockade. Monotherapy with the CTLA-4 antagonist ipilimumab resulted in a significant increase in overall survival of patients with metastatic melanoma in two large randomized trials, and is now one of the most promising immunotherapeutic agents (179, 180). In our pre-clinical studies, CTLA-4 blockade acts synergistically with RT to induce an abscopal response to RT in murine models of poorly immunogenic breast cancer and colon cancer (105). Importantly, these studies demonstrated that oligofractionation of RT

(8 Gy × 3) was more effective at inducing an abscopal response than a single large fraction of 20 Gy or more fractionated treatment (6 Gy × 5). We are currently testing this approach in an ongoing phase I/II clinical trial for patients with metastatic non-small cell lung cancer (ClinicalTrials.gov identifier: NCT02221739) and in a phase III, randomized trial for patients with metastatic melanoma (ClinicalTrials.gov identifier: NCT01689974). In the lung cancer study, patients with at least two measurable sites of disease are treated with 30 Gy in five consecutive fractions to one metastatic site with concurrent ipilimumab (3 mg/kg) administered intravenously every three weeks for four cycles starting within 24 h of the first fraction of RT. The same treatment is administered in the melanoma study but half of the patients are randomized to treatment with ipilimumab alone. The primary endpoints are the safety of the combined therapy and presence of an abscopal response in measurable metastatic sites on follow-up PET/CT, determined by immune-related response criteria using the modified WHO criteria.

Clinical trials using this same combination, but with a different treatment schedule and RT regimen have been recently published. An open-label phase I/II trial for men with metastatic castration-resistant prostate cancer tested escalated doses of ipilimumab from 3 mg/kg up to 10 mg/kg in 33 patients with or without a single 8 Gy dose directed at one to three osseous metastases. The highest dose of ipilimumab was well tolerated and an additional 34 patients were treated with concurrent radiation with only 25% of patients demonstrating progressive disease (181). To further test this treatment approach, a double-blind, randomized multi-center trial was conducted including 799 men with castration-resistant prostate cancer who progressed on docetaxel (182). Patients were treated with a single fraction of 8 Gy to one to five sites of osseous metastases and randomized to subsequent treatment with either 10 mg/kg of ipilimumab or placebo within 2 days of RT and continued every 3 weeks for up to four doses. The regimen was well tolerated but there was no difference in overall survival in the population as a whole. However, in subset analysis there was an improvement in overall survival of patients with a smaller burden of metastatic disease, demonstrated by alkaline phosphatase less than 1.5 the upper limit of normal, hemoglobin greater than 11 g/dL, and an absence of visceral metastases. While only limited clinical trial data are available in the published literature justifying a combined approach, this is an area of extremely active research (**Table 1**).

NEUTRALIZATION OF THE IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT

The immunosuppressive tumor microenvironment is one of the primary means of immune evasion by tumors, yet there are a few ongoing or completed studies combining this treatment approach with RT. The use of low-dose cyclophosphamide to deplete intratumoral Tregs is one example of this approach, and is sometimes used in combination with other immunotherapies. Another interesting study is testing tadalafil with RT. Tadalafil is a small molecule inhibitor of phosphodiesterase 5, which results in inhibition of myeloid-derived suppressor cell function and can target the suppressive myeloid response associated with hypofractionated radiation. An ongoing study for patients with locally advanced and borderline resectable pancreatic cancer is testing the combination of tadalafil with three fractions of 10 Gy delivered every other day to the primary tumor and grossly involved nodes, started after a 21 day cycle of gemcitabine, and patients with resected, stable, or responding disease continue on to receive an additional three cycles of gemcitabine. Like the other early phase studies, the primary endpoints of this study are feasibility and safety, with secondary endpoints looking at immune-correlates from blood and serum samples as well as immunohistology of resected tumor specimens and pathologic response rates (ClinicalTrials.gov identifier: NCT01903083).

CLINICAL APPROACHES: WHAT HAVE WE LEARNED?

Encouraging albeit preliminary results of combining RT and immunotherapy prompt a pause for reflection to take stock of what we have learned so far. Probably, the most promising results are from approaches enhancing the effector function of T-cells primed by RT. Combinations of RT with therapeutic vaccination have shown a more modest promise. The immunosuppressive effect of the tumor microenvironment is one potential reason for this. Vaccination, like RT, can induce priming of tumor reactive CTLs, but given alone it may not be able to overcome local immune suppression in the tumor. Future combinations of cancer vaccines with immunotherapeutics that enhance T-cell function or modulate the tumor microenvironment may prove to be more effective.

One approach that has not been adequately explored is the use of immunotherapeutics to modify the immune-suppressive tumor microenvironment prior to RT. RT has its own local effects on the tumor microenvironment, modifying regulatory lymphocytes, and recruiting new naïve myeloid cells such as DCs and TAMs. In established tumors, MDSCs are another targetable suppressive cell type that inhibit anti-tumor immunity. Modulation of the tumor microenvironment to counteract suppressive elements has the potential to act synergistically with RT to boost the systemic anti-tumor-immune response.

So far, several variables seem to be relevant to the success of combining immunotherapy and RT. Among them, dose and fractionation, site of irradiation, and sequencing with the selected modality deserve further discussion. Dose and fractionation are important factors in the immunogenicity of RT. Pre-clinical data suggests that when combined with CTLA-4 antibody antagonists, 8 Gy in three fractions or 6 Gy in five fractions are superior to standard fractionation or a single dose of 20 Gy (105). The underlying mechanism that explains the difference in immune effect among different dose and fractionation schedules is unclear, but these schedules are supported by the recent clinical reports of impressive abscopal effects after palliative RT to a single metastatic site in malignant melanoma (9.5 Gy × 3) and non-small cell lung cancer (6 Gy × 5) (101, 102).

The target site of RT may be another important consideration when combining RT with immunotherapy. Pre-clinical models are less instructive here, since most models involve radiation to tumors implanted into the subcutaneous tissue. However, when reviewing the clinical reports of abscopal effects, these were observed after irradiation targeting visceral metastases (97–99, 183–188).

The timing of RT relative to immunotherapy is another important consideration. This question has not been addressed

Table 1 | Active clinical trials testing the combination of ipilimumab and radiotherapy.

Clinicaltrials.gov identifier	Disease site	Design	Phase	Primary outcome measure	Radiation dose/ timing	Institution(s)
NCT01557114	Melanoma (stage III/IV)	1 arm: ipi with RT	I	Maximum tolerated dose	9, 15, 18, 24 Gy in three fractions with concurrent ipi	Gustave Roussy
NCT01996202	Melanoma (locally advanced or unresectable)	Two cohorts: (A) resected high-risk patients or (B) neoadjuvant, locally advanced	I	Safety and tolerability	No data provided	Duke University
NCT01565837	Melanoma (oligometastatic but unresectable)	1 arm: ipi with SRT	II	Safety and tolerability	SRT one to five lesions with third cycle of ipi	Comprehensive cancer centers of Nevada
NCT01703507	Melanoma (brain metastases)	Two arms: (A) ipi with WBRT or (B) ipi with SRS	I	Maximum tolerated dose	(A) WBRT weeks 1 and 2 (B) SRT week 1. Ipi delivered weeks 1, 4, 7, 10	Thomas Jefferson University
NCT01449279	Melanoma (stage IV)	One arm: ipi with RT	I	Safety	Pallitive RT within 2 days of ipi	Stanford
NCT01689974	Melanoma (stage IV)	Two arms, randomized: ipil ± RT	II	Tumor response	6 Gy × 5 given on consecutive treatment days starting on day 1 with Ipi on day 4	New York University
NCT01497808	Melanoma (metastatic)	One arm: ipi with SRT	I/II	Dose-limiting toxicity	SRT 1 lesion prior to ipi	University of Pennsylvania
NCT01970527	Melanoma (stage IV)	One arm: SRT before ipi	II	Immune-related response, toxicity and survival	3 fractions of SRT between days 1–13 followed by ipi	University of Washington/NCI
NCT01935921	Head and neck (stage III–IVB)	One arm: ipi, cetuximab and RT	I	Safety and tolerability	IMRT 5 days a week for 7 weeks with cetuximab and ipi at week 4 for 3, 21 day courses	NCI
NCT01711515	Cervical cancer (stage IB–IVA)	One arm: ipi, cisplatinum and RT	I	Safety and tolerability	Standard of care chemoradiation followed by 4, 21 day cycles of ipi within 2 weeks	NCI
NCT02107755	Melanoma (metastatic)	One arm: ipi followed by SRT	II	Progression-free survival	Ipi weeks 1, 4, 7, 10 with SRT two to three fractions on week 5–6	Ohio State Comprehensive Cancer Center
NCT02115139	Melanoma (brain metastases)	One arm: ipi followed by WBRT	II	One year survival	Ipi weeks 1, 4, 7, 10 with WBRT between cycles 1 and 2	Grupo Español Multidisciplinar de Melanoma
NCT01860430	Head and neck (stage III–IV)	One arm: IMRT with cetuximab and dose escalating ipi	II	Maximum tolerated dose	IMRT weeks 2–8 (70–74 Gy), Cetuximab weeks 1–8, ipi weeks 1, 5, 8, 11, 14	University of Pittsburgh/NCI
NCT02097732	Melanoma (Brain Metastases)	Two arms: (A) SRT followed by ipi (B) ipi then SRT then ipi	II	Progression-free survival	(A) SRT followed by 4 cycles ipi (B) 2 cycles of ipi then SRT then 2 cycles ipi	University of Michigan Cancer Center

Ipilumimab(ipi); Radiation Therapy (RT); Sterotactic Radiotherapy (SRT); Stereotactic Radiosurgery (SRS); Whole Brain Radiotherapy (WBRT); Intensity Modulated Radiation Therapy (IMRT); National Cancer Institute (NCI).

thoroughly in the pre-clinical models. In studies combining CTLA-4 blockade with RT using a mouse model of breast cancer, the antibody was administered at different time-points with the best abscopal response seen when the first dose of antibody was given during RT (105). Similarly, the patient with non-small cell lung cancer who experienced an abscopal effect had

received concurrent ipilimumab and radiation (102). Yet, the reported abscopal effect in a patient with metastatic melanoma occurred after long-term treatment with ipilimumab prior to RT (101).

Tumor burden and the associated degree of immunosuppression also play an important role in selection of the best candidates for trials combining radiation and immunotherapy. Metastatic tumor burden correlates with immune suppression, probably both as a marker of a weakened immune system and as an active player in systemic immune dysfunction (189, 190). The combination of ipilimumab with RT in men with castration-resistant prostate cancer resulted in a survival benefit only in patients with smaller burdens of metastatic disease, demonstrated by alkaline phosphatase less than 1.5 the upper limit of normal, hemoglobin greater than 11 g/dL, and absence of visceral metastases (182). Perhaps future trials should initially focus on patients with more limited metastatic disease.

Prior conventional therapy may also impact the results of immunotherapy trials. Many chemotherapeutic regimens cause myelosuppression, which depletes the very cells that are necessary for an effective immune response (191). However, some chemotherapeutic agents can cause immunogenic cell death and promote anti-tumor immunity (192). Also, despite the anti-tumor-immune promoting effects of RT, prior irradiation may lead to modification of the tumor microenvironment leading to a more immune-tolerant phenotype (113, 193). The net effect of these prior treatments is not clear, but it is likely to have an impact on the immune system and on the effectiveness of cancer immunotherapy.

Even something as fundamental as defining appropriate clinical endpoints is undergoing a critical re-appraisal, determining the best way to monitor the immune response to these combinations of immunotherapy and RT is an unresolved question. Specific immune responses are notoriously difficult to identify and track since every tumor has a unique complement of mutations and every patient has a unique MHC haplotype for presenting tumor antigens. As a surrogate to immune response and an alternative to the traditional RECIST criteria used to measure the effect of cytotoxic therapy, Wolchok et al. have introduced the immune-related response criteria (194, 195). These criteria take into account the mixed nature of clinical responses to immunotherapy, with some lesions responding while other lesions remain stable or even appear to progress. Importantly, overall survival and toxicity profiles, with their impact on quality of life, have emerged as the main clinical outcomes for immunotherapy. In some trials of immune monotherapy, most notably with sipuleucel-t, no objective response was observed; however, there was a significant improvement in overall survival (196). Multidisciplinary efforts to define optimal immunomonitoring are currently ongoing.

CONCLUSION

Ten years ago our group reported the first pre-clinical studies of the systemic anti-tumor effects of RT in combination with modern immunotherapy (104), after providing an immunological explanation for the abscopal effect (104). Now, a decade later, there are over 50 ongoing and published clinical trials combining RT and immunotherapy for the treatment of cancer, with more studies in the pipeline. Future directions may combine multiple approaches to immunotherapy that augment the effect of RT on anti-tumor T-cell priming as well as contribute to other steps of immune rejection (197). Many questions remain with regards to the optimal way to harness ionizing radiation in combination with immunotherapy, and how to best select patients for this approach, determining the most appropriate clinical characteristics, tumor pathology, and stage. Despite all of these challenges, the burgeoning interest in the combination of immunotherapy and RT will provide exciting new insights and avenues to explore as we continue our quest to harness patients' innate ability to eliminate evasive tumor cells.

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The Optimal Partnership of Radiation and Immunotherapy: from Preclinical Studies to Clinical Translation

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The Optimal Partnership of Radiation and Immunotherapy: from Preclinical Studies to Clinical Translation

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The main role of the immune system is to restore tissue homeostasis when altered by pathogenic processes, including neoplastic transformation. Immune-mediated tumor rejection has been recognized as an extrinsic tumor suppressor mechanism that tumors need to overcome to progress. By the time a tumor becomes clinically apparent it has successfully escaped immune control by establishing an immunosuppressive microenvironment. Ionizing radiation applied locally to a tumor alters these tumor-host interactions. Accumulating evidence indicates that standard therapeutic doses of radiation have the potential to recover tumor immunogenicity and convert the tumor into an *in situ* personalized vaccine. Radiotherapy induces an immunogenic tumor cell death promoting cross-presentation of tumor-derived antigens by dendritic cells to T cells. In addition, radiotherapy stimulates chemokine-mediated recruitment of effector T cells to the tumor, and cellular recognition and killing by T cells that is facilitated by upregulation of major histocompatibility antigens, NKG2D ligands, adhesion molecules and death receptors. Despite these effects, radiotherapy alone is only rarely capable of generating enough proinflammatory signals to sufficiently overcome suppression, as it can also activate immunosuppressive factors. However, our group and others have shown that when combined with targeted immunotherapy agents radiotherapy significantly contributes to a therapeutically effective anti-tumor immune response. To illustrate this partnership between radiation and immunotherapy we will discuss as an example our experience in preclinical models and the molecular mechanisms identified. Additionally, the clinical translation of these combinations will be discussed.

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INTRODUCTION

The primary role of the immune system is to protect against infectious agents, a function that has been successfully exploited by the development of many vaccines that prevent diseases. However, the immune system is also responsible for the maintenance of tissue homeostasis with important implications for many chronic diseases including cancer. In cancer, the immune system plays a dual role as an enabler to cancer development and progression and as an extrinsic tumor suppressor mechanism. While the purpose of inflammatory responses is to restore homeostasis, incomplete resolution of inflammation leads to chronic tissue stress, a maladaptive response that can promote genomic instability and cancer progression (*1, 2*). Conversely, the genomic instability associated with neoplastic transformation leads to the generation of neoantigens recognized by T cells (*3*), and to the expression of stress-induced ligands on cancer cells, for example members of the family of NKG2D ligands, which are recognized by natural killer (NK), γδ T cells and effector CD8 T cells (*4, 5*). Unscheduled cell death and local alterations in the stroma associated with tissue invasion generate degraded extracellular matrix components (e.g., heparin sulfate, hyaluronan), and other damage-associated molecular pattern (DAMP) molecules that act as danger signals and activate antigen-presenting cells by binding to Toll-like receptors (TLRs) (*6*). Overall, incipient tumors invariably attract the attention of the immune system, which is often successful at completely removing them. This process is known as the elimination phase of the tumor immuno-editing theory (*7*). Since complete elimination is not always successful, surviving cancer cells that have acquired the ability to evade immune recognition or suppress the anti-tumor response can emerge under the pressure of the immune system. The result is that by the time a tumor is clinically detectable it has usually become resistant to immune-mediated rejection (*8*). In fact, escape from immune-mediated control is now considered a hallmark of cancer (*9*). Importantly, many tumors co-exist with a concomitant anti-tumor T-cell response that has been shown to be associated with a better prognosis (*10–12*), providing evidence of tumor cell plasticity and of immune

escape. The recognition of this active process implies the possibility to intervene therapeutically and restore the ability of the immune system to hinder tumor progression or even cause its regression, even in the setting of overt metastatic disease. This is demonstrated by the clinical success of checkpoint inhibitors that, at least in a subset of patients, block negative regulatory pathways of T cells and recover an effective immune rejection (*13*).

However, an anti-tumor immune response that is powerful enough to control a tumor when it re-emerges by blocking immunosuppressive mechanisms or providing cytokines or other immune stimulatory factors is possible only in a minority of patients (*14*). In addition, while tumor types such as melanoma and renal cell carcinoma have been shown to respond to different immunotherapeutic interventions, most other solid tumors are refractory. Novel agents targeting the programmed death-1 (PD-1)/PD ligand-1 (PD-L1) pathway have enhanced the rates of durable tumor response to 38% used alone and >50% when used in combination with anti-CTLA-4 in metastatic melanoma, and shown activity in other cancer types (*15–19*), but overall the majority of patients with advanced cancer do not respond to immunotherapy alone.

Multiple obstacles hinder the priming and activation of anti-tumor T cells, their recruitment to the tumor site as well as their function, resulting in a formidable challenge to effective tumor rejection (*20, 21*). Ionizing radiation therapy has been known for a long time to cause inflammation in a dose-dependent manner, a side effect that oncologists have tried to minimize by manipulating fractionation and avoiding as much as possible the inclusion of normal tissue in the field of radiation. The appreciation of the potential benefits of the radiation-induced proinflammatory response has only recently emerged (*22, 23*). Work by several groups has identified molecular changes in the tumor microenvironment that contribute to conversion of the tumor into an *in situ* vaccine [reviewed in ref. (*24*)]. Radiation has been demonstrated to promote both, the priming and effector phases of the anti-tumor immune response. Priming results from the induction of an immunogenic tumor cell death by radiation (*25, 26*). In addition, radiation contributes to the effector phase by inducing chemokines and cytokines to recruit effector T cells to the tumor, and through the upregulation of major histocompatibility complex class I (MHC-I), adhesion molecules, death receptors and NKG2D ligands that enable recognition and elimination of cancer cells that have been damaged, but have survived the cytocidal effects of radiotherapy (*27–31*). The contribution of radiation-induced anti-tumor T cells to the response of the irradiated tumor, initially proposed by Stone and colleagues (*32*) is increasingly recognized (*33, 34*). Nevertheless, in most cases these responses are insufficient to result in an immune response capable to achieve systemic tumor control. Interestingly, the latter has been reported occasionally in patients undergoing radiotherapy to one site and responding at tumor sites outside of the radiation field, a phenomenon known as the abscopal (*ab-scopus*, away from the target) effect (*35*). Data in experimental models and patients suggest that the abscopal effect occurs when the anti-tumor immune response is sufficiently activated (*36, 37*). When we first made this observation in a preclinical model (*36*), it seemed reasonable to hypothesize that combining radiation with immunotherapy would provide the optimal therapeutic partnership to achieve immune-mediated systemic tumor control (*23*). Here we review our experience with the different combinations of radiation and immunotherapy tested so far.

Mouse Models of Cancer

To test whether local radiotherapy could induce an abscopal effect when combined with immunotherapy, we employed two main experimental settings that were designed to mimic both early and late metastatic disease (Fig. 1). The 4T1 mammary carcinoma is a poorly immunogenic and highly metastatic tumor. Circulating tumor cells are found within a week from implantation of 4T1 cells subcutaneously, and within a few weeks mice die of lung metastases outgrowth (*38*). The subcutaneous tumor was treated with local radiotherapy once it became palpable, 12–14 days post-implantation. At this time surgical resection of the tumor does not lead to a significant reduction in lung metastases (*39*) and, therefore, inhibition of lung metastases indicates an abscopal effect on visceral metastases rather than reduced dissemination from the irradiated tumor.

Another experimental setting that mimics more advanced metastatic disease with multiple detectable tumor nodules was employed for mouse carcinomas without (67NR and MCA38) or with low (TSA) ability to spontaneously metastasize when cells are injected subcutaneously (*38, 40, 41*). The cancer cells were injected at two separate sites in contralateral flanks, and radiotherapy was delivered to one nodule, mimicking the palliative use of radiation in metastatic disease.

We also tested the role of radiotherapy in the GL261 mouse model of high-grade glioma implanted intracranially. While this tumor type does not spread outside of the brain, it often recurs locally due to the highly infiltrative nature, a behavior that is reproduced in the mouse by GL261 cells (*42*). In this model we tested if immunotherapy could improve the response to whole brain radiotherapy (WBRT) and increase survival, a critical end point for this rapidly fatal tumor model.

Combination of Local Radiotherapy with a Dendritic Cell (DC) Growth Factor

Dendritic cells are professional antigen-presenting cells (APCs) with the unique ability to cross-present antigens from dying cells and activate T-ell responses (*43*).

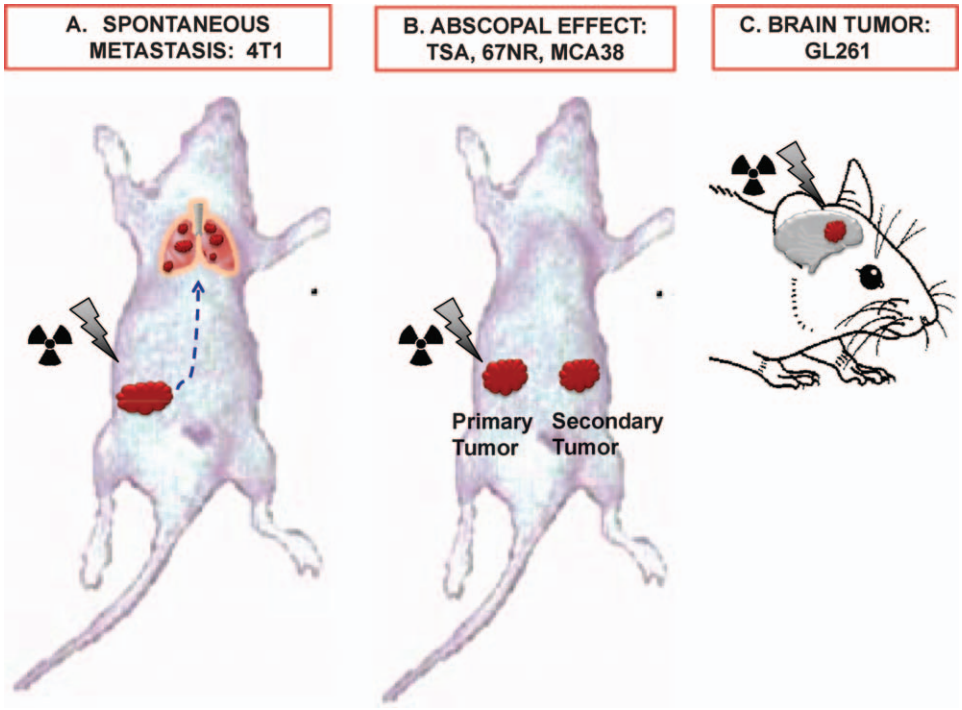

FIG. 1. Mouse models used to test combinations of radiotherapy and immunotherapy. Synergistic interaction between radiotherapy and immunotherapy were studied *in vivo* using 5 transplantable murine tumor models of breast (4T1, TSA, 67NR), colon (MCA38) and brain (GL261) malignancies. Panel A: 4T1 cells spontaneously metastasize from the ''primary'' subcutaneous tumor by the vascular route to the lungs. Outgrowth of lung metastases is responsible for death of the animals. Radiotherapy given to the primary tumor once it becomes palpable does not inhibit lung metastases. Panel B: TSA and 67NR are BALB/c-derived tumors. MCA38 is derived from C57BL/6 mice. Irradiation of one subcutaneous tumor module, by itself, does not affect the growth of another identical tumor outside of the radiation field. Panel C: GL261 cells are derived from C57BL/6 mice and grow with infiltrative borders when implanted stereotactically in the brain of syngeneic mice.

Therefore, the suboptimal function of DC in tumor-bearing hosts may be a critical barrier to induction of therapeutically effective anti-tumor T cells by radiotherapy. To overcome this barrier we treated mice bearing the mammary carcinoma 67NR with local radiation and Flt3-ligand (Flt3-L), a growth factor that improves DC numbers and function (*44*) (Fig. 2). Radiation by itself was unable to induce an abscopal effect, despite the fact that 67NR is a relatively more immunogenic tumor compared to 4T1 and TSA. Flt3-L did not have any significant effect by itself on tumor growth, but led to an abscopal effect when combined with radiotherapy (*36*). Expansion of tumor-specific $CD8^+$ T cells able to kill 67NR cells was detected only in mice receiving the combination of radiation and Flt3-L, and T cells were required for the abscopal effect. Overall, data support the concept that radiation generates an *in situ* vaccine by inducing an immunogenic tumor cell death but DC are required to uptake and present the released antigens. In the absence of optimally fit DC the immune response does not develop. This concept has received further support by the results of several studies showing that DC growth factors or injection of DC into irradiated tumors leads to development of anti-tumor T-cell responses (*45–47*).

Combination of Local Radiotherapy with a TLR7 Agonist

Toll-like receptors are a family of receptors expressed by innate immune cells that sense the presence of infectious agents and cellular damage by binding to a variety of pathogen-associated molecular pattern (PAMPs) and DAMPs molecules (*48*). Triggering of TLRs leads to activation of the type I interferon (IFN) and NFkB pathways resulting in production of IFN and proinflammatory cytokines, which enhance DC maturation and antigen presentation ability. Therefore, a variety of synthetic TLR agonists are under investigation as promising immunotherapy agents (*49*). Radiation induces the release from dying tumor cells of high-mobility group protein B1 (HMGB-1) which acts as a DAMP and binds to TLR4 (*25*). However, the ability of radiation to induce sufficient proinflammatory signals to optimally stimulate DC maturation is limited (*22*), suggesting that it could be complemented by administration of a TLR agonist. In support of this hypothesis, intratumoral delivery of the TLR9 agonist CpG has been shown to increase tumor response to radiation (*50*).

We chose to test the TLR7 agonist imiquimod (IMQ), which can be applied topically, in a mouse model of cutaneous breast cancer metastasis. The choice was

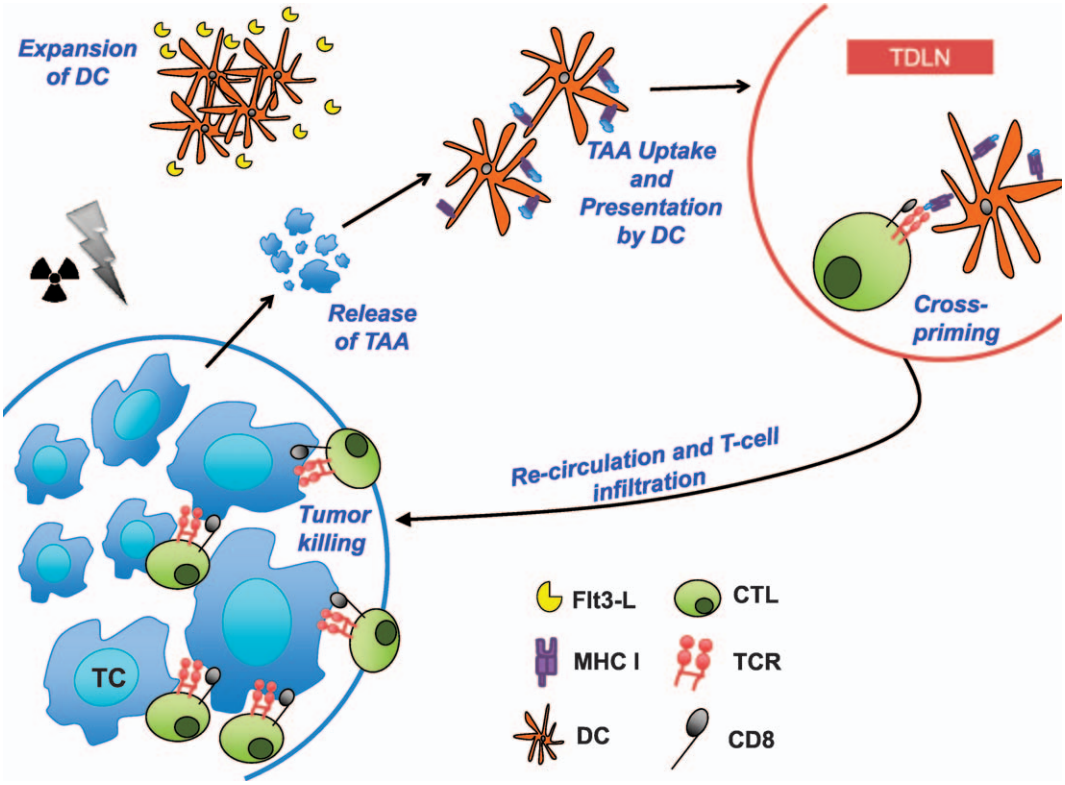

FIG. 2. Combination of radiotherapy and Flt3L. Ionizing radiation promotes cross-priming of anti-tumor T cells by inducing release of tumor-associated antigens (TAA) from tumor cells (TC). Dendritic cells (DC), which are expanded by administered FLt3L, uptake and process the TAA and present them as complexes with major histocompatibility (MHC) molecules. TAA-loaded DC travel to the tumor-draining lymph nodes (TDLN) where they activate naïve $CD8^+$ T-cells to become cytotoxic T-lymphocytes (CTL). Tumor-specific CTLs are recruited to the tumor where they kill tumor cells.

motivated by the fact that we had evidence of some activity of IMQ in breast cancer patients (*51*), and that it is FDA approved for topical treatment of some early skin cancers and known to have limited toxicity. IMQ was applied on the skin above TSA mammary carcinoma growing subcutaneously in mice 3 times/week. As single agent, IMQ caused increased tumor infiltration by DC, $CD8^+$ and $CD4^+$ T cells and slower tumor growth, an effect that was dependent on $CD8^+$ T cells (*52*). However, tumors kept growing despite treatment with IMQ. In contrast, when tumors were treated with local radiation given in 3 fractions of 8 Gy together with topical IMQ the majority of tumors showed complete regression. Like IMQ, radiation alone slowed tumor growth but did not induce complete regression. Importantly, in mice bearing two tumors, application of IMQ to the irradiated tumor induced an abscopal effect, which was enhanced by application of IMQ also on the tumor outside of the radiation field (*52*). Priming of tumor-specific T cells was confirmed in the lymph nodes draining the tumors treated with radiation and IMQ. In addition, IMQ-treated tumors showed increased expression of intercellular adhesion molecule-1 (ICAM-1) and MHC class I, suggesting that IMQ can sensitize tumor cells to rejection by $CD8^+$ T cells which are optimally activated and primed by the combination of radiation and IMQ. Thus, radiation and IMQ synergize in inducing tumor regression by multiple mechanisms, some of which are distinct but others may be overlapping (Fig. 3). Interestingly, the anti-tumor immune response elicited by radiation and imiquimod was not long lasting in all mice. In some mice tumors recurred after a variable tumor-free interval. Recurrence was reduced by administration of a single low-dose cyclophosphamide, which decreased IL-10 and Treg cells, suggesting a need to overcome immunosuppressive mechanisms to achieve long-term tumor control (*52*).

Combination of Local Radiotherapy with Checkpoint Receptor Blockade

Multiple pathways and mechanisms tightly regulate the activation of $CD4^+$ and $CD8^+$ T cells, resulting in productive immune responses that can be rapidly turned off once the offending agent has been cleared. This exquisitely orchestrated regulation is mediated by an array of costimulatory and coinhibitory or checkpoint receptors expressed by T cells (*53*). CD28 is a key costimulatory receptor that delivers a second signal required for T-cell activation in addition to T-cell receptor (TCR) engagement. CD28 binds to B7-1 and B7-2 molecules expressed on APC and induces interleukin (IL)-2 production culminating in robust T-cell proliferation.

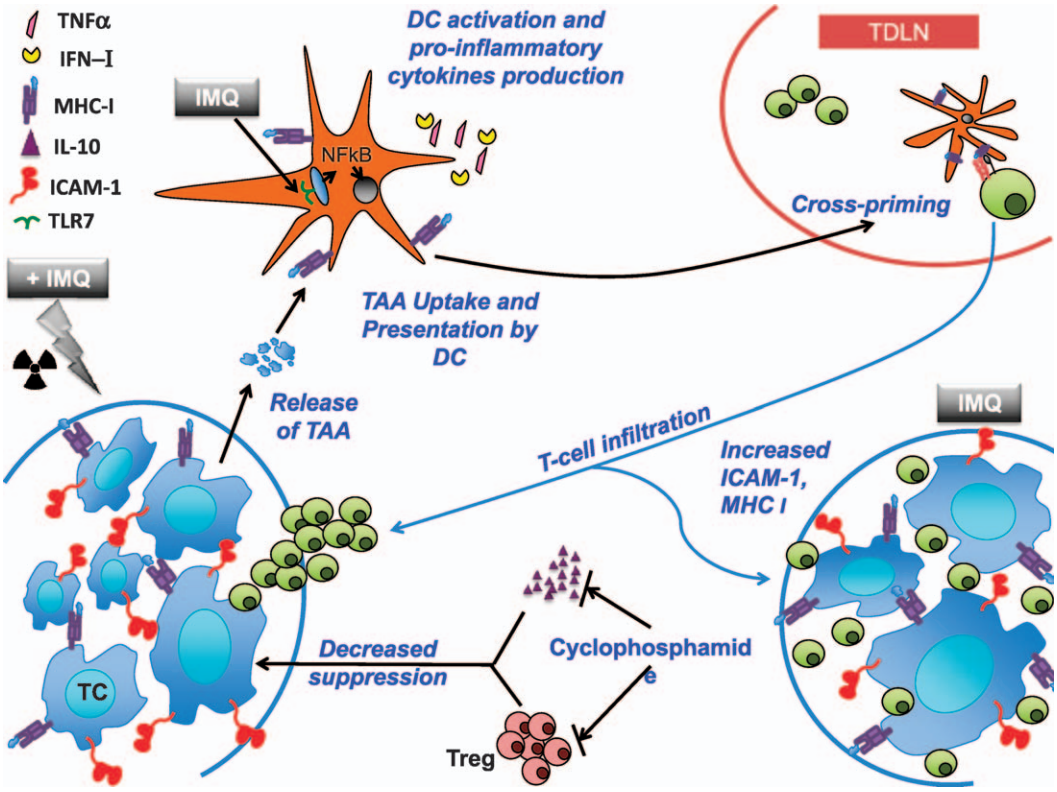

FIG. 3. Combination of radiotherapy and TLR7 agonist. Imiquimod stimulates production of type I IFN and proinflammatory cytokines by a toll-like receptor (TLR)-7 expressed mainly in DCs. This results in enhanced maturation and activation of DC and improved cross-priming of anti-tumor T cells to TAA released by radiation. Primed CTLs migrate to irradiated and nonirradiated tumors. Here imiquimod-induced upregulation of ICAM-1 and MHC-1 molecule on tumor cells (TC), increases their susceptibility to killing by CTL. Administration of cyclophosphamide reduces IL-10 levels and Treg numbers and results in a more sustained anti-tumor T cell response.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is the prototypical checkpoint receptor, limiting T-cell activation and proliferation to prevent autoimmunity (*54*). Induced shortly after TCR signaling is triggered through cognate interaction with peptide-MHC, CTLA-4 is rapidly recruited to the immune synapse where it binds to B7-1 and B7-2 with greater affinity than CD28, thus outcompeting CD28 when co-stimulatory molecules are present in limiting amounts (*55*). In addition, CTLA-4 constitutively expressed on regulatory T cells (Treg) exerts its inhibitory function by removing B7-1 and B7-2 from the surface of APC (*56*). Chronic antigen exposure in the context of cancer leads to T cell exhaustion and increased expression of CTLA-4 on effector T cells. Together with reduced costimulation of APC and increased Treg presence, this promotes tolerance of anti-tumor T cells (*54*). The importance of this checkpoint receptor in cancer has been clearly demonstrated by the ability of monoclonal antibodies (mAb) against CTLA-4 to induce effective anti-tumor immunity (*57*). However, the response is limited in the clinic to a subset of patients and in pre-clinical tumor models is seen only in relatively immunogenic tumors (*54, 57*).

We hypothesized that radiotherapy could convert tumors unresponsive to anti-CTLA-4 into responsive ones by its ability to convert the irradiated tumor into an immunogenic hub. This was first tested in the poorly immunogenic 4T1 carcinoma model (Fig. 1). While radiation given to established tumors delayed significantly the growth of the subcutaneous irradiated tumor, it did not reduce lung metastases and median survival of treated mice was comparable to control cohorts (*58*). As expected, anti-CTLA-4 mAb did not show any anti-tumor activity by itself, but synergized with radiation improving control of the irradiated tumor and inhibiting lung metastases. This response was mediated by induction of anti-tumor $CD8^+$ T cells and led to a significant extension of mice survival (*58*). The therapeutic synergy of the combination of local radiotherapy and anti-CTLA-4 was confirmed in two additional tumor models, TSA and MCA38, syngeneic to mice of different genetic background (Fig. 1). Interestingly, we found that the radiation regimen employed was a critical determinant of the ability of radiation to synergize with anti-CTLA-4 mAb and induce anti-tumor T cells able to mediate an abscopal effect (*59*). A fractionated regimen of 8 Gy × 3 given on consecutive days was the most effective, while a

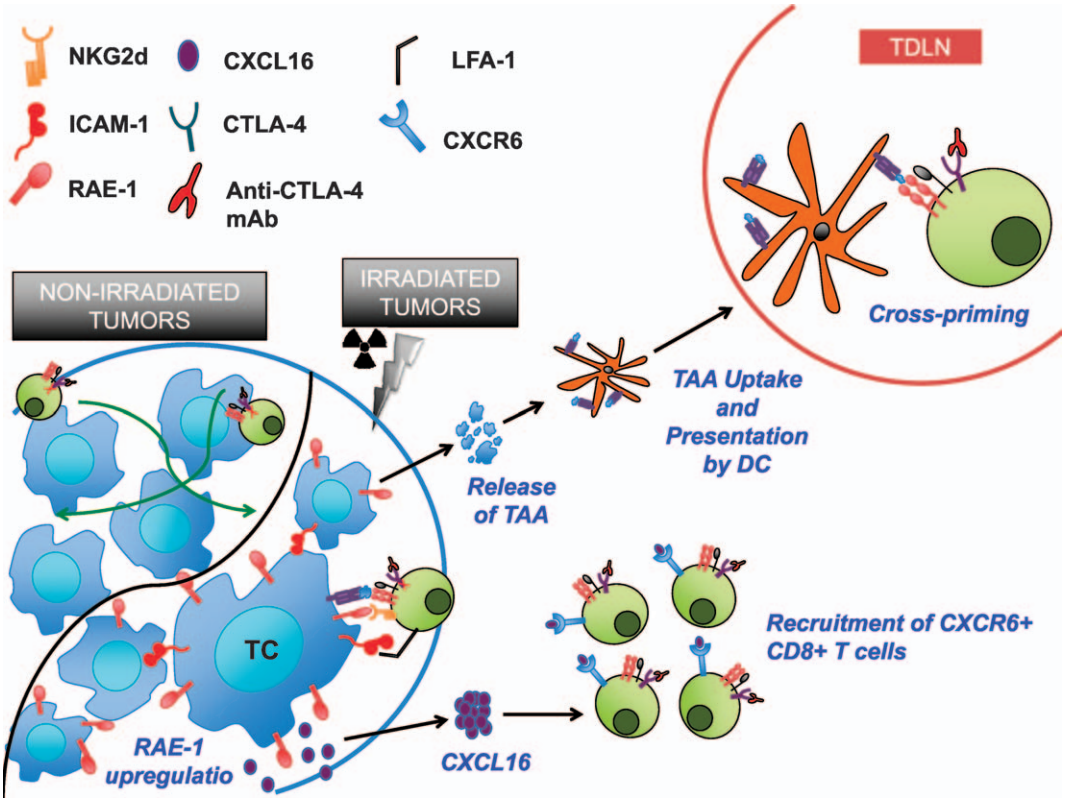

FIG. 4. Combination of radiotherapy and anti-CTLA-4 antibody. Multiple mechanisms underlie the cooperative effects of ionizing radiation and CTLA-4 checkpoint blockade. The uptake and presentation by DC of TAA released from dying cells promotes cross-priming of tumor-specific T cells, which is mediated by engagement of T-cell receptor (TCR) by MHC/antigen complexes and lymphocyte function-associated antigen 1 (LFA-1), and is enhanced by blocking CTLA-4. Primed $CD8^+$ effector T cells are recruited to the tumor by radiation-induced CXCL16. Inside the tumor, upregulation of RAE-1 promotes immune synapse formation between the cancer cells and $NKG2D^+$ CTLs leading to cancer cell killing and tumor regression.

single large dose (20 Gy) was unable to induce an abscopal effect in combination with anti-CTLA-4. The molecular bases for this difference between radiation regimens are currently being investigated.

Mechanisms responsible for the synergy of radiation with anti-CTLA-4 mAb were further investigated in the 4T1 model (Fig. 4). We found that regressing tumors were infiltrated by $CD8^+$ T cells expressing the activation marker CD69 and chemokine receptor CXCR6 (*27*). CXCR6 was responsible for recruitment of tumor-infiltrating lymphocytes (TILs) to the irradiated 4T1 tumors, since reduced numbers of $CD8^+$ TILs were seen in $CXCR6^{-/-}$ mice. Consistently, the ligand for CXCR6, the chemokine CXCL16, was significantly upregulated in 4T1 tumor cells by radiation, both in vitro and in vivo. Chemotaxis assays confirmed that CXCL16 released by irradiated 4T1 cells attracted activated $CD8^+$ T cells towards the tumor cells. Importantly, $CXCR6^{-/-}$ mice that have T cells unable to sense CXCL16 showed impaired tumor control after treatment with radiation and anti-CTLA-4 mAb (*27*). Collectively, these studies implicate the key role of CXCR6/CXCL16 interactions in driving radiation-induced recruitment of effector anti-tumor T cells in the 4T1 model. We found that CXCL16 was induced by radiation in several human breast cancer cells, as well as in other mouse cells, including prostate and colorectal carcinoma, suggesting that enhanced recruitment of activated T cells may be a common effect of radiotherapy (*27, 60*).

Additional analysis of the dynamic behavior of $CD8^+$ TILs by two photon laser scanning microscopy (TPLSM) revealed a molecular interaction that is critical for tumor rejection in mice treated with radiation and anti-CTLA-4 (*30*). Stable interactions between effector $CD8^+$ T cells and target tumor cells are required for the formation of an immune synapse and tumor cell killing (*61*). Radiation-induced expression of the NKG2D ligand retinoic acid early inducible-1 (RAE-1) on tumor cells was required to promote the formation of such immune synapse. TILs moved faster without stopping in contact with target tumor cells in mice treated with anti-CTLA-4 or radiation as monotherapy, while the opposite was seen when the two modalities were combined. Blocking the interaction of NKG2D receptor expressed on effector $CD8^+$ T cells with RAE-1 induced by radiation on tumor cells abrogated the therapeutic response to anti-CTLA-4 treatment in 4T1 tumor-bearing mice (*30*). These data suggest that NKG2D

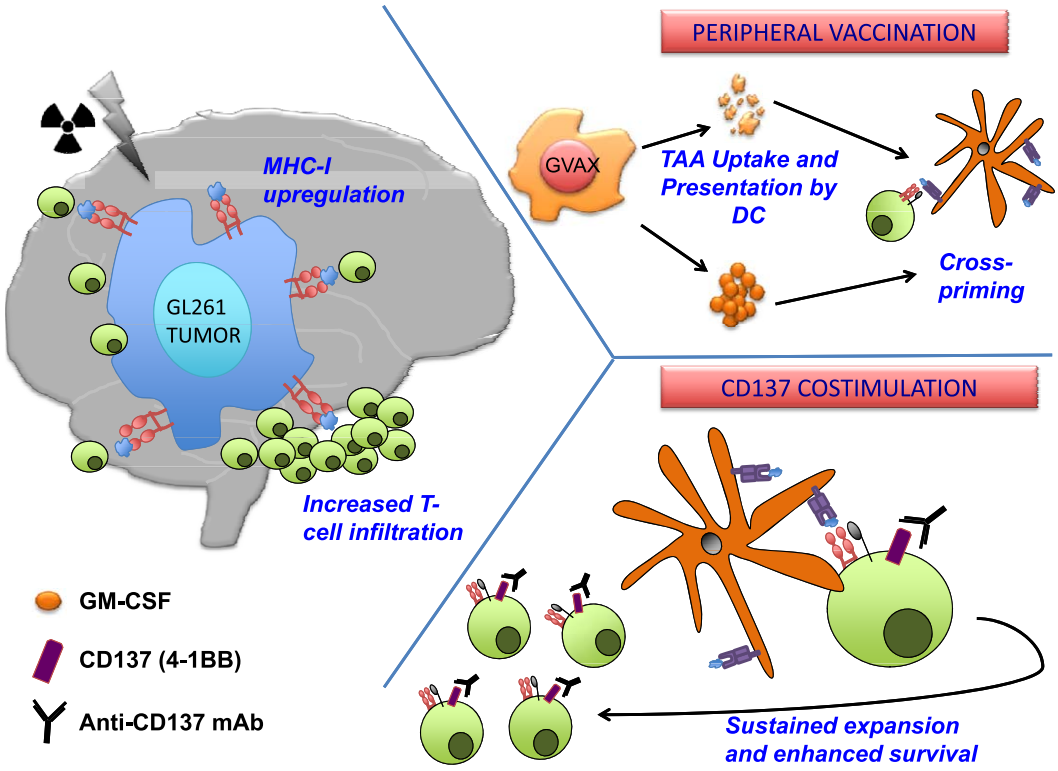

FIG. 5. Combination of whole brain radiotherapy (WBRT) and immunotherapy. Ionizing radiation promotes immune recognition of GL261 glioma cells by upregulating tumor expression of MHC class I molecules and promoting influx of effector T-cells in the tumor microenvironment. Robust anti-tumor T cells sufficient to induce tumor regression are generated by either vaccination with autologous tumor cells modified to produce GM-CSF (GVAX), or by promoting expansion and survival of anti-tumor T cells primed by endogenously released antigens after WBRT by agonistic mAb to the co-stimulatory CD137/4-1BB receptor.

ligand expression may be a determinant of tumor response to anti-CTLA-4 immunotherapy, and provides a novel molecular mechanism for the synergy between radiotherapy and CTLA-4 treatment (*62*).

Combination of Local Radiotherapy with Vaccination

Granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes the maturation and antigen presenting ability of DCs, which play a central role in T-cell priming. Vaccination with autologous tumor cells modified to secrete GM-CSF was shown to be effective at inducing a robust and sustained anti-tumor immune response in preclinical models and some clinical trials (*63, 64*). In 2006, we tested whether peripheral vaccination with autologous tumor cells transduced with GM-CSF could enhance the effectiveness of WBRT in the GL261 glioma model (*65*). Response of established intracranial GL261 glioma was significantly improved when WBRT was combined with peripheral vaccination, resulting in increased overall survival. Mice with intracranial tumors typically succumbed within 33 days from initial implantation, and survival was modestly increased by monotherapy with either WBRT alone (median survival of 55 days) or vaccine alone (median survival of 45 days). On the other hand, 80% of animals given WBRT + vaccine survived more than 75 days, and most survivors rejected a secondary tumorigenic GL261 inoculum.

In vitro, irradiation (4 or 6 Gy) of GL261 cells enhanced expression of MHC class I molecules, increasing their susceptibility to killing by $CD8^+$ T cells (*65, 66*). *In vivo* WBRT given in 2 fractions of 4 Gy induced strong surface expression of MHC class I on invading glioma cells. WBRT also enhanced tumor infiltration by $CD4^+$ and $CD8^+$ T cells, suggesting that radiation was effectively enhancing tumor rejection by T cells activated by the vaccine (Fig. 5).

Combination of Local Radiotherapy with Co-stimulation by CD137/4-1BB

CD137 (4-1BB, TNFRSF9) is a member of the tumor necrosis factor receptor (TNFR) superfamily which is expressed following activation by T cells, natural killer (NK) cells, neutrophils, monocytes and DCs (*67*). CD137 ligation enhances T cell proliferation, functional maturation and production of cytokines. CD137 provides a strong survival signal especially for $CD8^+$ T cells, primarily by upregulation of anti-apoptotic Bcl-2 molecules. Importantly, anti-CD137 mAb have shown anti-tumor activity in several preclinical models (*68*).

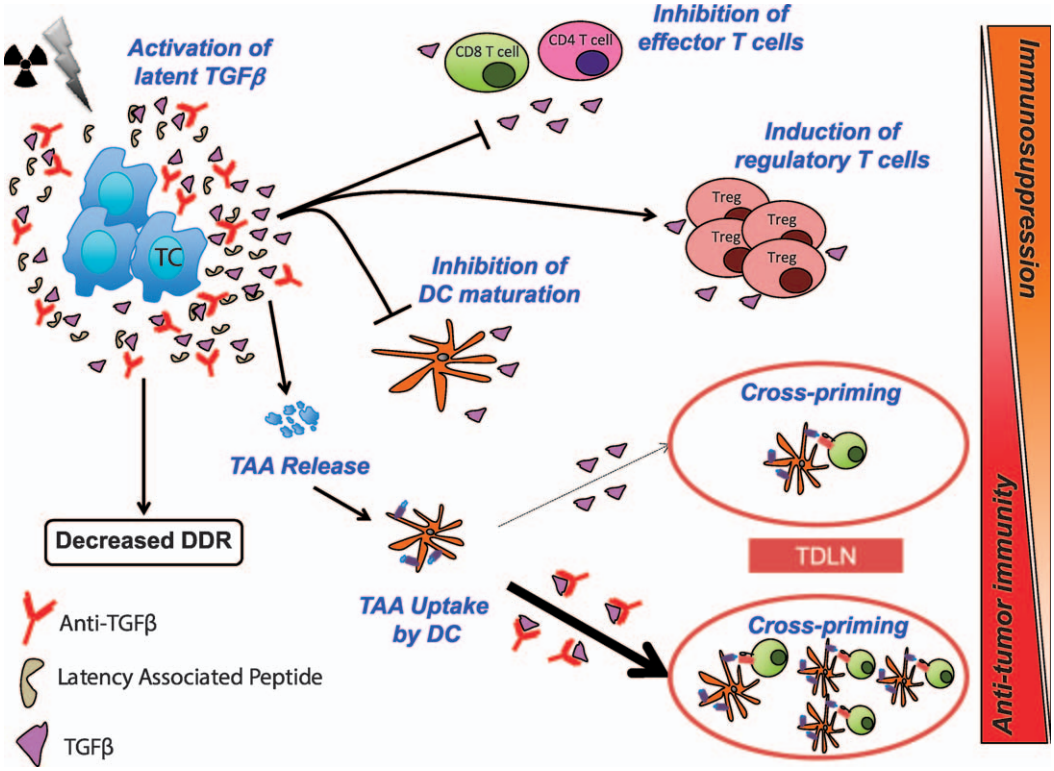

FIG. 6. Combination of radiotherapy and TGFβ blockade. Ionizing radiation kills tumor cells releasing TAA, but also activates the immunosuppressive cytokine TGFβ by promoting its dissociation from the latency-associated peptide (LAP). TGFβ inhibits the antigen-presenting function of DC and the differentiation of T cells into effectors, while promoting their differentiation into regulatory T cells. TGFβ neutralization by anti-TGFβ mAb enhances antigen-presentation by DC, promoting cross-priming and acquisition of effector function by anti-tumor T cells, leading to a shift from immunosuppression to anti-tumor immunity. Neutralization of TGFβ also increases radiosensitivity of tumor cells by inhibiting the DNA damage response (DDR).

To determine if radiation induced an immune responses to an intracranial tumor that could be enhanced by CD137 co-stimulation, 15 days after intracranial GL261 implantation mice were given WBRT in two fractions of 4 Gy and anti-CD137 mAb starting on the day after the last irradiation (*69*). The combination of WBRT and anti-CD137 mAb improved significantly survival with a median of 114 days compared to 31 days in the untreated control group, 37 days with WBRT alone and 42 days with anti-CD137 alone, thereby indicating a synergistic effect elicited by the combination treatment. The majority of animals treated with WBRT and anti-CD137 became long-term survivors and showed anti-tumor memory responses able to reject a secondary challenge of viable GL261 cells. A massive increase in TILs was seen in mice treated with WBRT and anti-CD137, which was more pronounced for $CD8^+$ T cells (36-fold over the background in untreated mice), while WBRT alone and anti-CD137 alone caused only a 4- to 6-fold increase in TILs (*69*). Tumor-specific production of IFNγ by spleen T cells was markedly increased only in mice treated with WBRT and anti-CD137. Collectively, the data is consistent with the interpretation that radiation induces priming of anti-tumor T-cells that require additional costimulatory signals to acquire effector functions and to persist (Fig. 5). In addition, WBRT facilitates tumor rejection in the brain by improving T cell recruitment and infiltration. Therefore, complementary effects of radiation and CD137 costimulation seem to underlie the synergy between these two treatments.

Combination of Local Radiotherapy with TGFβ Neutralization

As discussed above, tumors able to progress have a highly immunosuppressive microenvironment that allows them to escape immune-mediated control. One key mediator of immunosuppression is the cytokine transforming growth factor (TGF)β which is produced by cancer cells and by some immune cells with regulatory function such as Treg and myeloid-derived suppressor cells (*70*). Importantly, radiation activates latent TGFβ (*71, 72*). In addition to suppression of T cell and DC function, TGFβ enhances multiple processes that support tumor progression and resistance to radiation, including angiogenesis, epithelial to mesenchymal transition and DNA damage response (*73*). Therefore, blocking TGFβ in the context of radiotherapy may yield multiple benefits (Fig. 6). In support of this notion, we have shown that antibody-mediated neutralization of TGFβ increased radiation sensitivity of 4T1 cells by

impairing DNA damage repair, and significantly increased tumor growth delay in response to single and fractionated radiation *in vivo* (*74*). Importantly, our recent data indicate that TGFβ is a key regulator of radiation-induced anti-tumor immune responses (Vanpouille-Box *et al*., manuscript in preparation). Overall, these data provide a strong rationale for testing the combination of radiotherapy and strategies to block TGFβ in cancer patients.

Clinical Translation

Radiation and chemotherapy are currently used to palliate patients with metastatic or recurrent local-regional disease. While long lasting remissions are rare, most patients derive some measurable benefit from either treatment. With the advent of novel immunotherapies the possibility of sustained anti-tumor immune responses is emerging (*75*). However, to date it is unknown whether anti-cancer immunity developed from radiation in conjunction with immunotherapy can lead to tumor regression and long lasting systemic effects. Thus, based on our preclinical data, we designed several clinical studies to detect the abscopal effects of radiation and immunotherapy and assess for sustained immunological responses.

Because the clinical responses to immunotherapy do not exactly mirror the responses to chemotherapy, several criteria to standardize assessment of immunologic responses to immunotherapies were proposed (*76*). In each of the clinical trials we are conducting, the sites of disease for each patient are assessed with clinical/radiological evaluation, including PET/CT, at baseline and after treatment. Whenever possible, additional serial blood draws and/or biopsies are obtained for in depth immunological assessment.

GM-CSF

The findings from our experiments in the preclinical models highlighted above suggest that adding a treatment that increases DC numbers and function to radiation can induce effective anti-tumor immunity. We hypothesized that the induction of tumor cell death by concomitant chemotherapy and radiation to a specific metastatic site may enhance tumor immunogenicity by promoting cross-priming and eliciting anti-tumor T-cell responses in patients. Similarly to Flt3L, GM-CSF has the potential to enrich the DC compartment and could improve anti-tumor immunity elicited by concurrent chemotherapy and radiotherapy. A clinical trial in patients with metastatic solid tumors was designed to test this hypothesis (*77*).

Patients who had demonstrated no change or early progression after single agent chemotherapy were eligible: they were maintained on the same systemic treatment but radiation to a site of metastatic disease and GM-CSF were added. The main endpoint for this exploratory study was to assess whether the abscopal response achieved in the preclinical model could be detected in patients. Radiation was given to a total dose of 35 Gy in 10 fractions. After completing the first week of irradiation, patients were given GM-CSF (125 μg/m^2 subcutaneously) administered daily for 2 weeks. Abscopal responses were assessed, thereafter, by measuring nonirradiated target lesion(s) clinically and radiologically. An abscopal response was detected in 30% of the patients (*78*).

Imiquimod

Based on the preclinical data indicating that the combination of local radiotherapy and imiquimod induces anti-tumor immune responses that are active both locally and systemically, we designed a single arm, open label Phase I/II clinical trial for breast cancer patients with multiple cutaneous metastasis, which is ongoing (http://clinicaltrials.gov/show/NCT01421017).

At trial entry, all skin metastases are outlined and photographed (including visible/palpable borders). Topical imiquimod is applied to all skin metastases while radiotherapy is given to one area only. The lesion to be irradiated is chosen by the radiation oncologist to limit normal tissue toxicities, especially if the patient was previously irradiated. This site is treated to a total dose of 30 Gy (with either electrons and/or photons) distributed in 5 fractions of 6 Gy delivered every other day. Responses are assessed in skin metastases treated with radiation and imiquimod and with imiquimod alone. Since many of these patients have additional metastases to internal organs, responses are also assessed radiologically in these untreated metastases. In some patients without detectable visceral metastases an area of skin is left untreated to measure the abscopal effect. Clinical and radiological assessment of untreated lesions is performed at week 9.

Fresolimumab

Our preclinical data with radiation and TGFβ neutralization suggest that the combination may act to radiosensitize tumor cells by reducing DNA repair mechanisms, while inducing anti-tumor immune responses. We hypothesized that similar effects may be clinically observed. Fresolimumab (GC1008) is a human mAb that neutralizes TGFβ and is being tested in early clinical trials for a few diseases, including cancer. The number of patients receiving GC1008 is small and, at this point, information regarding any possible clinical benefit remains limited.

Based on our preclinical work we designed a trial to combine Fresolimumab and radiation to one metastatic site in patients with mestastatic breast cancer, which is currently enrolling (http://clinicaltrials.gov/ct2/show/NCT01401062). Since the optimal dose of GC1008 to neutralize TGFβ in irradiated cancer patients is unknown, eligible patients are randomly assigned to two different doses of Fresolimumab, either Arm 1 (1 mg/kg of GC1008) or Arm 2 (10 mg/kg of GC1008). The antibody is administered intravenously every 3 weeks for a total of 5 infusions at the assigned dose. The chosen metastatic site receives conformal external beam

radiation 7.5 Gy per fraction, given every other day to a total of 22.5 Gy. The first lesion is irradiated at week 1 (radiation starts after 1st dose of GC1008), lesion 2 is irradiated at week 7. Patients are assessed for response by PET/CT imaging. Serial blood samples are collected to monitor changes in cytokines, lymphocytic and myeloid populations and to measure development of tumor-specific T cells.

Ipilimumab

In early 2011, ipilimumab (a humanized antibody to CTLA-4) was approved by the U.S. FDA to treat patients with metastatic melanoma (*79*). Since its approval, ipilimumab, when given occasionally in combination with radiation, has led to abscopal responses in some auspicious patients (*37, 80*). The most provocative abscopal response was reported in a patient that demonstrated radiographic evidence of disease progression, while on ipilimumab maintenance therapy. Growth of a paraspinal mass, which caused right-sided back pain, triggered the indication for palliative radiotherapy, administered concurrently with maintenance ipilimumab. The treatment resulted in regression of distant disease in the spleen and mediastinal lymph nodes. Interestingly, the therapeutic response temporarily correlated with an increase in antibody titers targeting NY-ESO-1 and other tumor associated antigens, an increase in $CD4^+$ T-cell and myeloid lineage activation, and a decline in the quantity of myeloid-derived suppressor cells, lending credence to the immunologic hypothesis of the abscopal effect (*37*). Encouraged by these anecdotal cases and based on our preclinical work, we designed a trial to test whether the combination of radiation and Ipilimumab can induce an anti-tumor immune response at the irradiated site capable to elicit immune-mediated abscopal effects. A phase I randomized trial tests ipilimumab immunotherapy with local radiotherapy in patients with metastatic melanoma who have at least two separate measurable sites of disease documented by CT scanning or MRI prior to entering the study (http://www.clinicaltrials.gov/ct2/show/NCT01689974). Patients are then randomized to either Arm A (ipilimumab alone) or Arm B (ipilumimab with radiation). Immune-monitoring includes T cell and B cell responses to melanoma associated tumor antigens.

CONCLUSIONS

Accumulating data in preclinical studies and clinical observations highlight the importance of this new area of investigation, aimed at identifying the most promising combinations of radiotherapy and immunotherapy for treatment of different cancers. This new application of radiotherapy has at least two important implications. The first is that it can change the role of radiation in metastatic disease from a palliative measure to one that has the potential to extend survival and perhaps even cure some patients. The second implication is that it requires a new partnership between radiation oncologists and immunotherapists in management of patients. The latter will be greatly facilitated by incorporating training in tumor immunology in the curriculum of residents training in oncology.

Noticeably, responses to immunotherapy occur even in heavily pretreated metastatic disease, providing a real new option for patients who would normally have exhausted available therapeutic possibilities.

The growing number of clinical trials testing combinations of radiotherapy and immunotherapy represents an outstanding example of translation form preclinical models to clinical studies. The immunological consequences of tumor irradiation not only provide a therapeutic opportunity, but also highlight the critical role of the tumor microenvironment as a determinant of the response to radiation. This improved understanding of the role of the immune system in response to radiation makes a compelling case for the use of immunocompetent animals for testing response to treatment in experimental conditions.

Overall, to assure the success of the use of radiation as a partner for immunotherapy it is critical to gain more insights into the mechanisms at play. Support for basic, translational and clinical studies in this field is required to deliver the promise of this new treatment strategy.

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